Towards the elucidation of the mechanism of the antibiotic activity of tamoxifen

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Towards the elucidation of the mechanism of theantibiotic activity of tamoxifen

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SUMMARY

Antibiotic resistance is increasingly a health and financial burden on the global population. Use and misuse of antibiotics has led to increased frequencies of antibiotic-resistant infections worldwide, leading to fatalities as well as greatly increasing healthcare costs. To combat this, researchers have done much work to expand to the field of antibiotics, delving back into old compounds and testing massive libraries of compounds with rapid screening techniques. Tamoxifen is one such compound that is primarily used as an anticancer agent, but displays many useful other characteristic, including antibacterial effects. However, the mechanism of the antibacterial effects of tamoxifen are poorly documented. My research was aimed at both improving the effectivity of tamoxifen as an antibacterial and elucidating the mechanism of action of tamoxifen.

CHAPTER 1. ANTIBIOTICS

Antibiotics have a relatively short history, with development beginning in the 1930's, slowly as the idea was adopted, exploding in the 1960's, and decelerating significantly to the present day as many natural sources of novel antibiotics are exhausted. A brief history of antibiotics, along with a survey of the majority of relevant antibiotic classes, their histories, mechanisms, and uses are presented in this chapter.

1.1 The discovery and development of antibiotics

In 1928, Alexander Fleming took a vacation from his work at St. Mary's, leaving behind Petri dishes with bacterial cultures. Upon his return, he found the plates a mess of contamination. As he sorted through and discarded the ruined cultures, he noticed that one plate had mold growing along the edge. Furthermore, there was a clear zone between the mold and the nearest bacterial colony. Ever the scientist, Fleming pursued an explanation to this phenomenon which culminated in the discovery of the first known antibiotic: penicillin[1]. From such odd beginnings, an entirely new branch of medicine developed. It took over a decade for penicillin to make the transition to clinical trials, where it was used to treat a patient with a staphylococcal infection in 1941. Although that patient died due to a paucity of the compound[2], penicillin was moved into large scale production in time for World War II. Fleming and the two scientists who isolated penicillin and developed the production process, Howard Florey and Ernst Chain, were awarded the Nobel Prize in 1945. This monumental success opened the floodgates which swept into the so-called "Golden Age" of antibiotics, a period from the 1950s to the late '60s which saw the discovery and clinical development of many antibiotics which remain relevant to this day[3]. The euphoria of innovation soon wore off as the problem of antibiotic resistance cropped up within a decade of the introduction

of penicillin. This predicament is one of the most concerning developments in the medical field and some have even speculated that should this dilemma continued unchecked it will lead to a world where antibiotics are ineffective, a regression of a century's worth of medical advances[4]. Antibiotic resistance is already responsible for tens of thousands of deaths worldwide, and significantly impacts the worldwide economy through increased treatment costs and hospital days, costing millions of man-hours[5]. Fortunately, awareness of this issue is high and research groups worldwide now strive to overcome resistance mechanisms and unearth or create novel antibiotics.

1.2 Major antibiotic classes

There are many classes of antibiotic classes which target various aspects of microbial cells. This section is arranged in an outward-in manner, beginning with antibiotics that affect the cell wall and moving to antibiotics with internal targets.

1.2.1 Antibiotics which target or affect the bacterial cell wall

The following antibiotic classes either directly interact with the bacterial cell wall or else act in some manner to inhibit or disrupt the growth of the bacterial cell wall.

1.2.1.1 Lipopeptides

Lipopeptides, as the name suggests, are cyclic peptides with attached lipid moieties. These antibiotics are used for skin and skin structure infections. They are highly effective against Gram-positive bacteria, including MRSA[6]. Daptomycin (Fig. 1) was the first clinically available lipopeptide. A fermentation product of *Streptomyces roseoporus*, it was first developed by Eli Lilly and Company under the name LY146032 in 1986. The rights were acquired by Cubist pharmaceuticals with clinical studies beginning in 1999[7]. Lipopeptides such as daptomycin display a unique mechanism (Fig. 2) of action among antibiotics; the lipid 'tail' of the lipopeptide

inserts into the cell membrane of the bacteria, causing rapid depolarization and ion efflux, leading to an arrest of DNA, RNA, and protein syntheses, ending in cell death[6]. Resistance has not been described in a laboratory setting, but a patient who displayed daptomycin-resistant *Staphylococcus aureus* bacteremia has been documented and there is growing concern over reports of more cases of daptomycin-resistance[8].

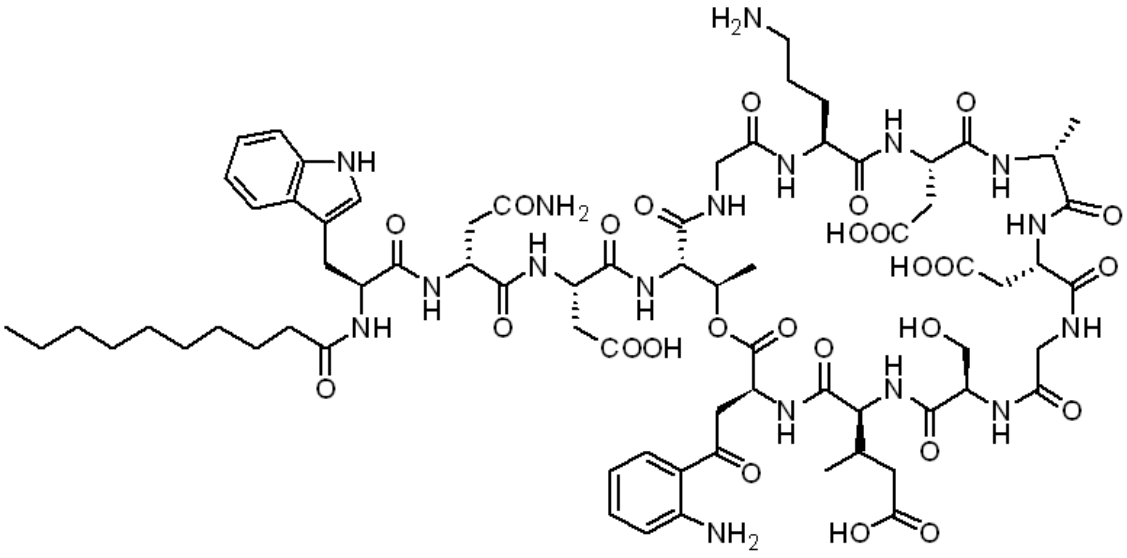


Figure 1. Structure of daptomycin

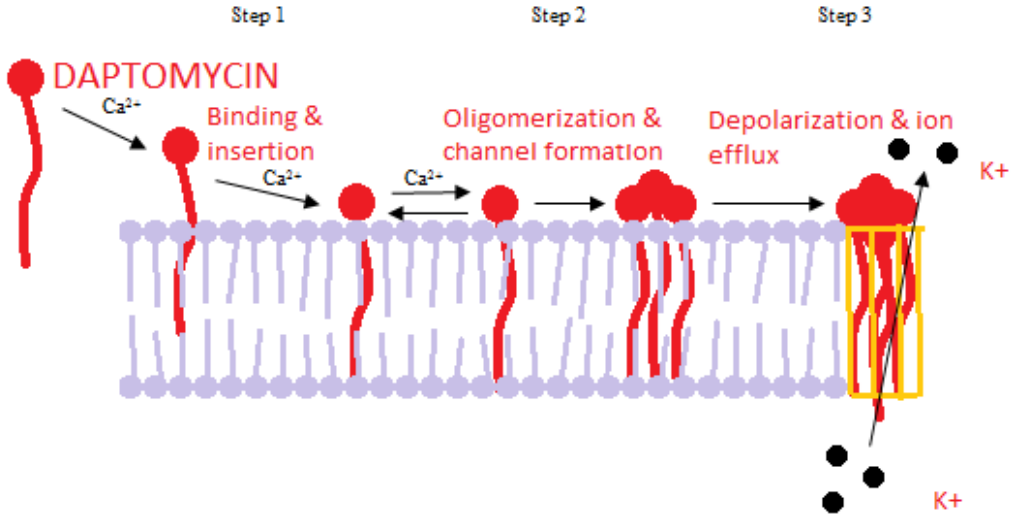


Figure 2. Mechanism of daptomycin.

1.2.1.2 Polymyxins

Polymyxins such as colistin (Fig. 3) are large, cyclic peptides with long hydrophobic tails derived from species *Bacillus polymyxa* which were first isolated in 1940[9]. Unfortunately, although polymyxins show excellent broad spectrum activity, they have several disadvantageous side effects, including relatively high neuro- and nephrotoxicity, limiting their clinical use to antibiotics of last resort, to be used only when mainline antibiotics have failed[10]. The primary use of polymyxins is in topological creams and ointments, for instance, generic "triple antibiotic ointment" contains polymyxin B. Polymyxins are surface active peptides, cationic agents which bind to the anionic cell membrane of bacteria, leading to a detergent effect which disrupts cell membranes (Fig. 4). The polymyxins have an especially high affinity for the lipid moiety of lipopolysaccharides, displacing magnesium and calcium ions[10]. Resistance to polymyxins is intrinsic in microbial strains which have alterations in lipid A which lead to lower binding. Acquired resistance generally takes the form of altered or displaced phosphate groups on the lipopolysaccharide, as well as changes in cell membrane composition[11].

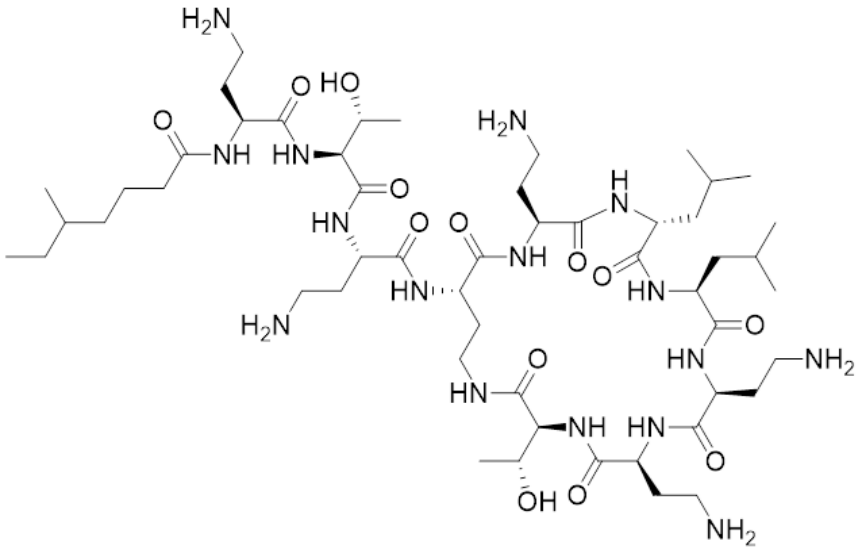


Figure 3. Structure of colistin

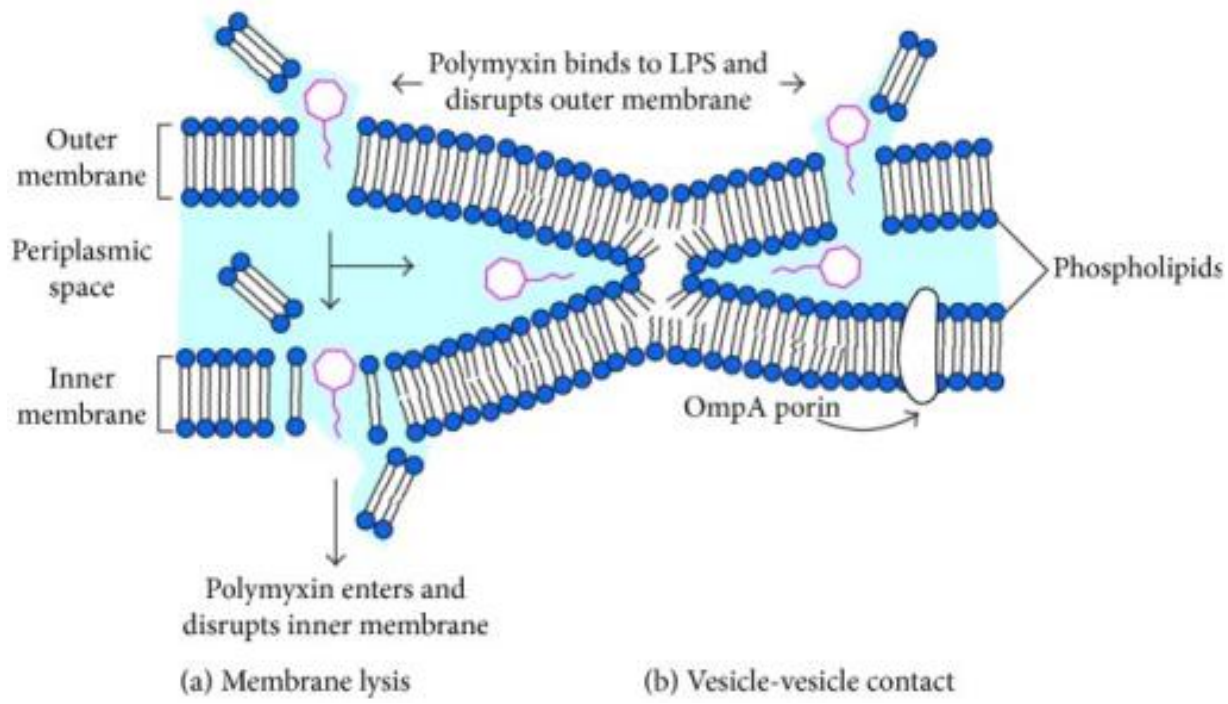


Figure 4. Mechanism of polymyxin action. Adapted with permission under open-access policy from [12]

1.2.1.3 Glycopeptides

Glycopeptides are glycosylated, cyclic, nonribosomal peptides, such as vancomycin and bleomycin. Vancomycin (Fig. 5) was first used in 1958 and thanks to its activity in patients who were refractive to other antibiotics, it received quick approval from the FDA. In the next few years, however, newer β-lactams emerged which had the same coverage as vancomycin and were considered to be less toxic, so vancomycin faded from the public eye. Recently it has experienced a resurgence due in large part to the necessity brought on by antibacterial resistance[13]. Glycopeptides in general have narrow-spectrum coverage, primarily used to treat Gram-positive bacteria, and also suffer from poor bioavailability and various deleterious side effects[14]. Glycopeptides, like β-lactams, inhibit cell wall synthesis. This is accomplished by binding to a peptidoglycan, the building blocks of the bacterial wall, where the peptidoglycan ends in consecutive alanine residues, called the D-Ala-D-Ala moiety, which is specific to bacterial cell

walls. Glycopeptides bind this moiety, sterically shielding it from modification and preventing further cell wall synthesis, leading to cell death[15]. Resistance genes such as VanA generate alternate moieties from D-Ala-D-Ala which allow for cell wall synthesis but restrict glycopeptide binding[16].

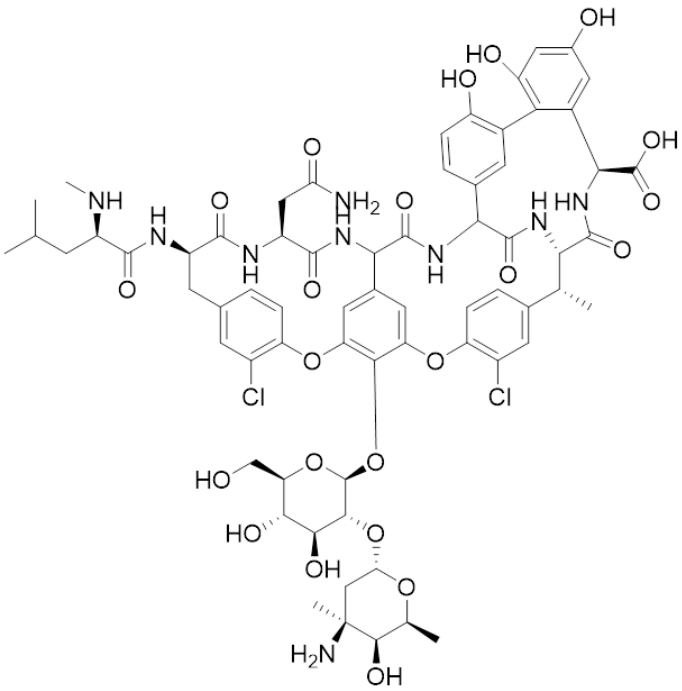


Figure 5. Structure of vancomycin

1.2.1.4 β-lactams

β-lactam (Fig. 6) is an umbrella term for any antibiotic that contains a β-lactam ring. This broad group includes penicillins, cephalosporins, monobactams, and carbapanems, to name a few. The general mode of inhibition for β-lactams is disruption of bacterial cell wall biosynthesis. This group of antibiotics is generally more effective on Gram-positive bacteria. β-lactams are the most widely used antibiotic group, constituting 50% of global antibiotic usage[17]. This section will

also discuss β-lactamase inhibitors, compounds which are used as a co-treatment with β-lactam antibiotics to overcome a common mode of resistance, β-lactamases. The method by which β-lactams inhibit cell wall synthesis is the binding of transpeptidases, which earns those proteins the alternative name of penicillin-binding proteins (PBPs), proteins which are bound by molecules containing β-lactams. The binding of these various proteins generally prevents a transpeptidation reaction which halts crosslinking of the peptidoglycans in the bacterial cell wall, leading to cell lysis. The exact mechanism is complex, and some penicillins do not inhibit transpeptidation to achieve cell lysis[18]. Recently, it was shown that rather than simply inhibiting transpeptidation, β-lactams "stimulate a deleterious futile cycle of cell wall synthesis and degradation by their target machineries that contributes to their lethal activity"[19]. In short, β-lactams induce a continual building and recycling of peptidoglycan, depleting the precursor pool but never achieving cross-linkage.

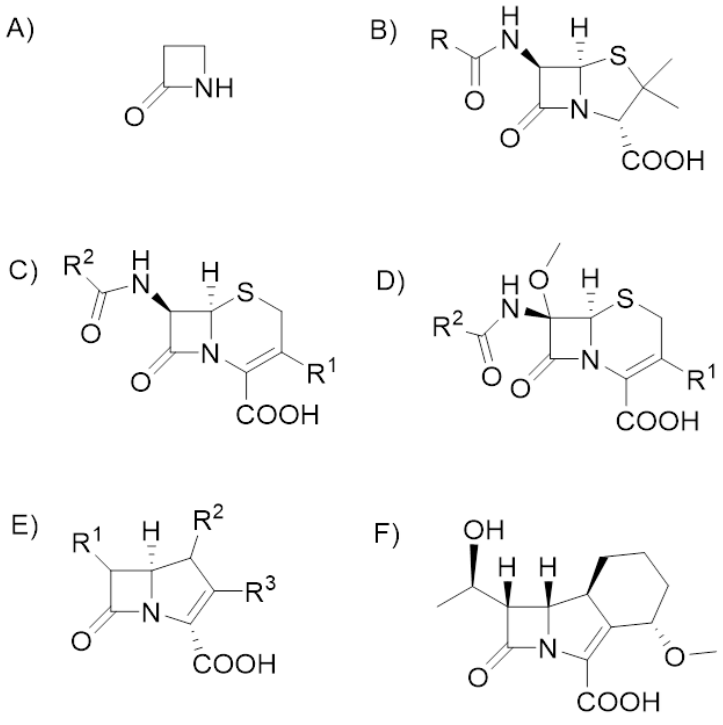


Figure 6. β-lactam cores. A) naked β-lactam ring B) penicillin C) cephalosporin D) cephamycin E) carbapenem F) trinem

1.2.1.4.1 Penicillins

As mentioned in the introduction, penicillin was the first antibiotic ever characterized. The penicillin class quickly grew and is one of the most substantive subclasses of antibiotics available. The penicillins all share three components: the β-lactam ring, an attached thiazoldine ring, and a side chain. This group includes penicillin, ampicillin, and methicillin. Natural penicillins are generally used to treat enterococci, streptococci, and staphylococci which do not present β-lactamases[20]. To treat bacteria which presented penicillinase, an enzyme which opens the β-lactam, rendering the drug inert, semi-synthetic derivatives of natural penicillins are used, such as methicillin, which presents a large side-chain to restrict access of the enzyme to the β-lactam ring[21]. This extends the antibacterial coverage to most staphylococcus, however it has also lead to one of the most prominent instantiations of antibacterial resistance – methicillin-resistant *Staphylococcus aureus* or MRSA. By modifying the side chain with an amine group, aminopenicillins were developed. These penicillins were the first to show activity against Gram-negative bacteria and include ampicillin and amoxicillin[20]. Finally, several penicillins have been developed which display a wide coverage, dubbed 'extended-spectrum penicillins, including carbenacillin and piperacillin. These penicillins show increased activity against *Pseudomonas sp.*[22]. Penicillins are widely used in primary care and are commonly co-administered with β-lactamase inhibitors.

1.2.1.4.2 Cephalosporins

Cephalosporins were first discovered by Guiseppe Brotzu in the late 1940s and were isolated in the early 1950s[23]. Although these compounds themselves were not particularly potent antibiotics, by removing the natural side chains and isolating the common core of a β-lactam ring condensed with a dihydrothiazine ring, referred to as 7-aminocephalosporanic acid (7-ACA),

development of more powerful cephalosporin generations was possible[24]. Cephalosporins show very broad activity and can be generally categorized by generation. The first generation, including Cephalexin and Cephradine, have relatively simple structures and generally have a methyl side chain at C3. They perform well against Gram-positive bacteria but are lacking with regards to Gram-negative bacteria. Second generation cephalosporins, such as Cefuroxime and Cefotiam introduced an α-iminomethoxy group at the C7 side chain, which increased resistance to β-lactamases, and the attachment of an aminothiazole ring to the C3 side chain, which increased binding affinity and antimicrobial activity[25]. The third generation, including Cefixime and Cefdinir, moved the iminothiazole group to the C7 position and utilized various groups at the 7α position to increase β-lactamase resistance[25]. The fourth generation improved activity against Gram-negative bacteria by adding a positively charged element to make the molecules zwitterionic, allowing them to diffuse through Gram-negative bacterial membranes more easily than their predecessors[25]. The latest generation, the fifth, is still young and as such only contains a handful of compounds, including Ceftobiprole and Ceftaroline. These compounds are the only cephalosporins effective against MRSA, both having their C3 side chain tailored specifically to bind to PBPs in MRSA[26]. Although these two compounds have been shown to be effective against MRSA, the rise of extended-spectrum β-lactamases has largely made cephalosporins useless in treating resistant bacteria[27].

1.2.1.4.3 Cephamycins

Cephamycins are structurally very similar to cephalosporins, but have a methoxy group at the 7α position. This group was discovered in 1972[28] and includes compounds such as cefoxitin and cefmetazole. These compounds exhibit a broad spectrum of activity and the methoxy group plays

a key role in increasing resistance to β-lactamases. As such, these compounds are highly effective against Gram-negative bacteria.

1.2.1.4.4 Carbapenems

Carbapenems share an almost identical core with penicillins, except that a carbon is substituted for the sulfur in the 5-membered ring which additionally has a degree of unsaturation. The first carbapenem isolated was theienamycin, isolated from *Streptomyces cattelya* with some difficulty, due to the compound's instability[29]. Thienamycin displayed an unusually broad antibacterial spectrum for a β-lactam, but its aforementioned instability made it unfit for clinical usage, as it shows a tendency to dimerize into an inert form as the concentration increases[29]. The first clinically viable carbapenem was imipenem, created by derivatizing the terminal amine to a formamidine. This compound showed activity against both Gram-positive and Gram-negative bacteria, as well as retaining activity against β-lactamase producers[30]. The carbapenem family is of particular importance for its activity against *Pseudomonas aeruginosa* and *Enterococcus sp* and is also boasts the broadest spectrum of the β-lactam family of antibiotics. In recent years, however, more and more cases of resistance to carbapenems have surfaced, reducing the utility of these potent drugs[31].

1.2.1.4.5 Monobactams

The monobactams, as the name suggests, are antibiotics with an unfused, lone β-lactam ring. First isolated in 1981, the only clinically used monobactam, aztreonam, was synthetically derived through a structure-activity relationship (SAR) study, where subtle iterations of a compound are synthesized to parse out a more effective molecule, of naturally occurring monobactams[32].

Monobactams are particularly effective against Gram-negative bacteria such as *Pseudomonas aeruginosa* but do not bind well to Gram-positive PBPs, limiting their use clinically.

1.2.1.4.6 Trinems

Trinems, or tribactams, are a new class of β-lactams which contain a tricyclic ring. They display broad spectrum activity against aerobic and anaerobic Gram-positive and Gram-negative bacteria, and are highly resistant to β-lactamases[33]. To the author's knowledge, no trinems are currently in use clinically.

1.2.1.4.7 β-lactamase inhibitors

β-inhibitors are compounds that, while not having much antibiotic activity on their own, impede or disrupt the bacterial enzymes which break β-lactam rings, thereby increasing the microbe's sensitivity to a β-lactam antibiotic which is used as a co-treatment. β-lactamase inhibitors may have a β-lactam core, as in the case of clavulanic acid, sulbactam, and tazobactam, or they may not, as in the case of avibactam. These inhibitors are usually used in the treatment of Gram-negative bacteria as the general mode of resistance for Gram-negative bacteria is the production of β-lactamases, whereas Gram-positive bacteria tend to exhibit resistance due to variant PBPs[34].

1.2.2 Antibiotics which target the ribosome

There are many different classes of antibiotics which bind to the ribosome and negatively affect protein translation, to varying degrees and through many different mechanisms.

1.2.2.1 Aminoglycosides

Aminoglycosides are compounds whose basic structure consists of one or more aminated sugars joined to a dibasic cyclitol; they display broad-spectrum antibiotic coverage[35]. The first aminoglycoside used clinically, streptomycin (Fig. 7), also carries the honor of being the second-most widely used antibiotic, after penicillin. In 1943, Albert Schatz joined the laboratory of Selman Waksman for his PhD studies. The Waksman lab at the time had identified several candidate microbes which secreted compounds that were effective against tuberculosis (TB). Schatz was able to isolate streptomycin, and clinical trials began in 1945. The treatment proved effective, winning Waksman the Nobel prize for the discovery, causing some controversy as Waksman refused to acknowledge Schatz' role in the work[36]. In the 1970's, many semisynthetic aminoglycosides were developed such as dibekacin and amikacin, but recently the development of new aminoglycosides has slowed considerably[35]. Aminoglycosides bind at the A-site in the bacterial ribosome. Although this binding does not prevent protein synthesis, it impairs the proofreading process, leading to the production of aberrant proteins and eventually cell death[37]. In *E. coli*, for example geneticin and paromomycin bind adjacent to a pair of adenines, A1492 and A1493, which will flip out from helix 44, signaling a codon-anticodon match. When there is a mismatch, the pair does not flip out, signaling the cell that a mismatch has occurred. When aminoglycosides are bound, the adenine sensor pair remains flipped out, signaling the cell to continue translation, essentially muting the mismatch warning system[38]. A primary means of antibacterial resistance to aminoglycosides is severely reduced uptake by means of membrane impermeabilization, which holds high clinical relevancy since it results in moderate resistance across all aminoglycosides[39]. Aminoglycosides are also susceptible to modification by *N*-acetyltransferases and *O*-nucleotidyltransferases, which make the compounds unsuitable for

ribosomal binding. This mode of resistance is more challenging in the long run, as many of these enzymes are encoded on transposable elements, making dissemination of resistance possible[37].

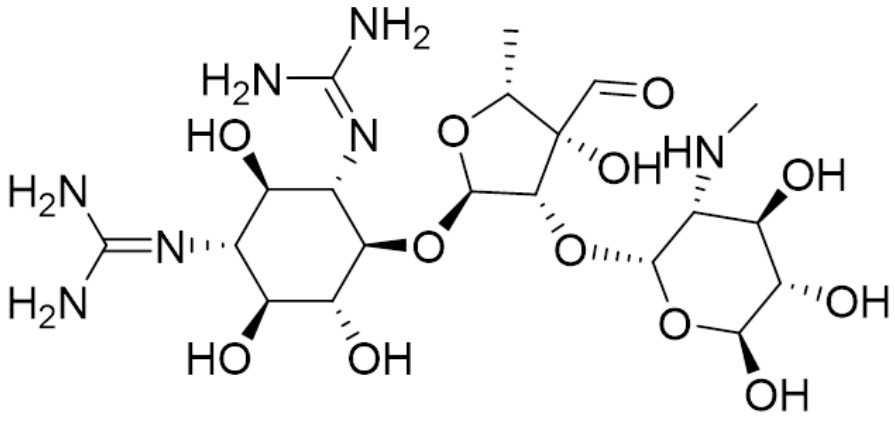


Figure 7. Structure of streptomycin

1.2.2.2 Tetracyclines

The tetracyclines are a group of compounds with a fused 4-ring system, generally recognized as a naphthacene core (Fig. 8). The first clinical usage of tetracycline was in 1948 when a 5-year old boy with a ruptured appendix faced complications after surgery. When the few other antibiotics available were ineffective, the boy's parents consented to allow the use of a previously untested compound, Aueromycin. The boy made a full recovery and tetreacycline compounds were recognized as an important new treatment[40]. Currently, tetracyclines are considered first-line treatments for a variety of diseases, including Rocky Mountain spotted fever and Lyme disease. This group of compounds shows broad-spectrum activity with little innate resistance, although acquired resistance has become an increasing issue in recent years. Tetracyclines act by inhibiting protein synthesis by binding to the 30S subunit and blocking incoming aminoacyl-tRNAs from binding, shutting down translation[41]. The binding pocket for the compound(Fig. 9) is thought to be composed of the S7 protein and several rRNA residues on the 16S subunit, although there are

other interactions revealed by footprinting experiments that suggest other possible binding modes[40]. Additionally, there are 'atypical' tetracyclines such as chelocarrin which disrupt cell membranes at higher concentrations in addition to attacking the ribosome[42], however these compounds display adverse side effects and are not used clinically[41]. The major mechanism of tetracycline resistance is efflux pumps, which reduce the intracellular concentrations of the compound to sub-inhibitory levels. The pumps are encoded on the mobile *tet* genes, and are spread horizontally[41]. A second mechanism of resistance is ribosomal protection proteins (RPPs), cytosol soluble proteins which displace tetracyclines from their binding sites in a GTP-dependent manner[43].

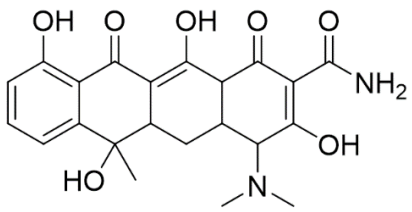


Figure 8. Structure of tetracycline

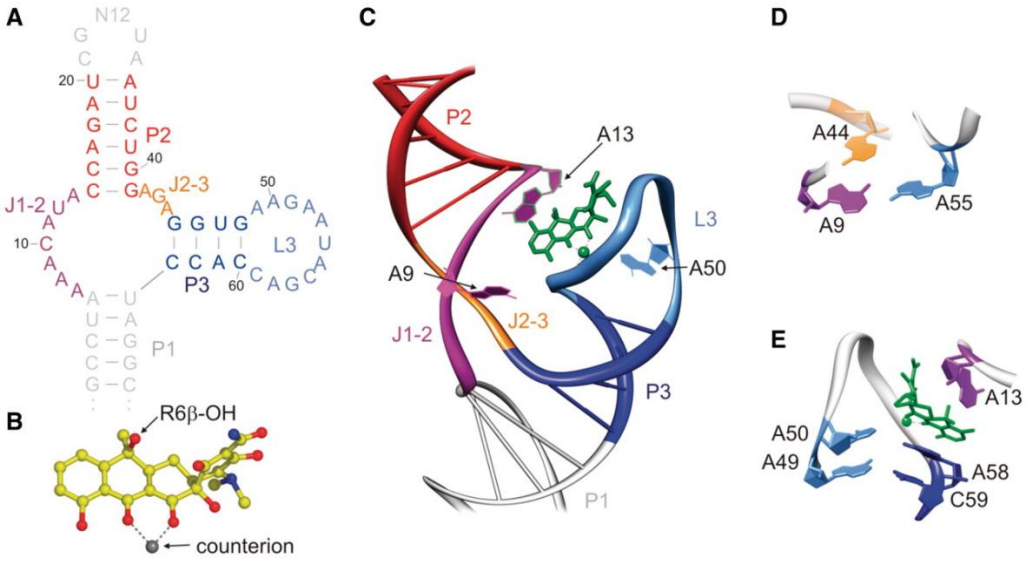


Figure 9. The tetracycline-binding aptamer. A) Secondary structure and C) crystal structure of the aptamer. Tetracycline shown in green. B) Tetracycline structure D) Base triplet between A9, A44, and A55. E) Location of A13 and A50 with respect to tetracycline. Adapted from with permission under open-access policy [44]

1.2.2.3 Amphenicols

The amphenicols, which include chloramphenicol and florfenicol, are a group of relatively small molecules with a phenylpropanoid core. Chloramphenicol (Fig. 10) was first isolated in 1949, and thanks to its simple structure, was the first antibiotic to be completely synthesized chemically[45]. It shows broad spectrum coverage but has deleterious side effects and as such is reserved for treatment only in cases where other antibiotics have proven ineffective. Amphenicols target the 50S ribosomal subunit, where they prevent chain elongation by inhibiting the peptidyl transferase center of the ribosome (Fig. 11). Specifically, amphenicols bind the A2451 and A2452 residues of the 23S rRNA and prevent peptide bond formation[46]. Enzymes such as acetyltransferases have been found to confer amphenicol resistance, such as in *Streptococcus haemolyticus* and *faecalis*[47]. Another mechanism of resistance, discovered in *Haemophilus influenza*, causes a decrease in amphenicol uptake due to a permeability barrier stemming from the loss of an outer membrane protein[48].

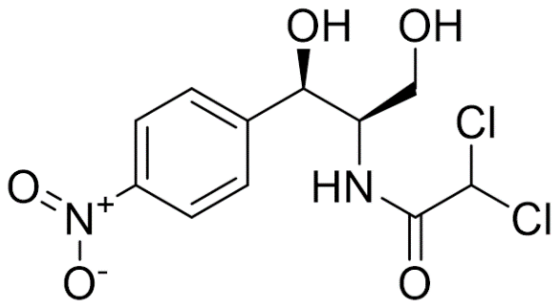


Figure 10. Structure of chloramphenicol

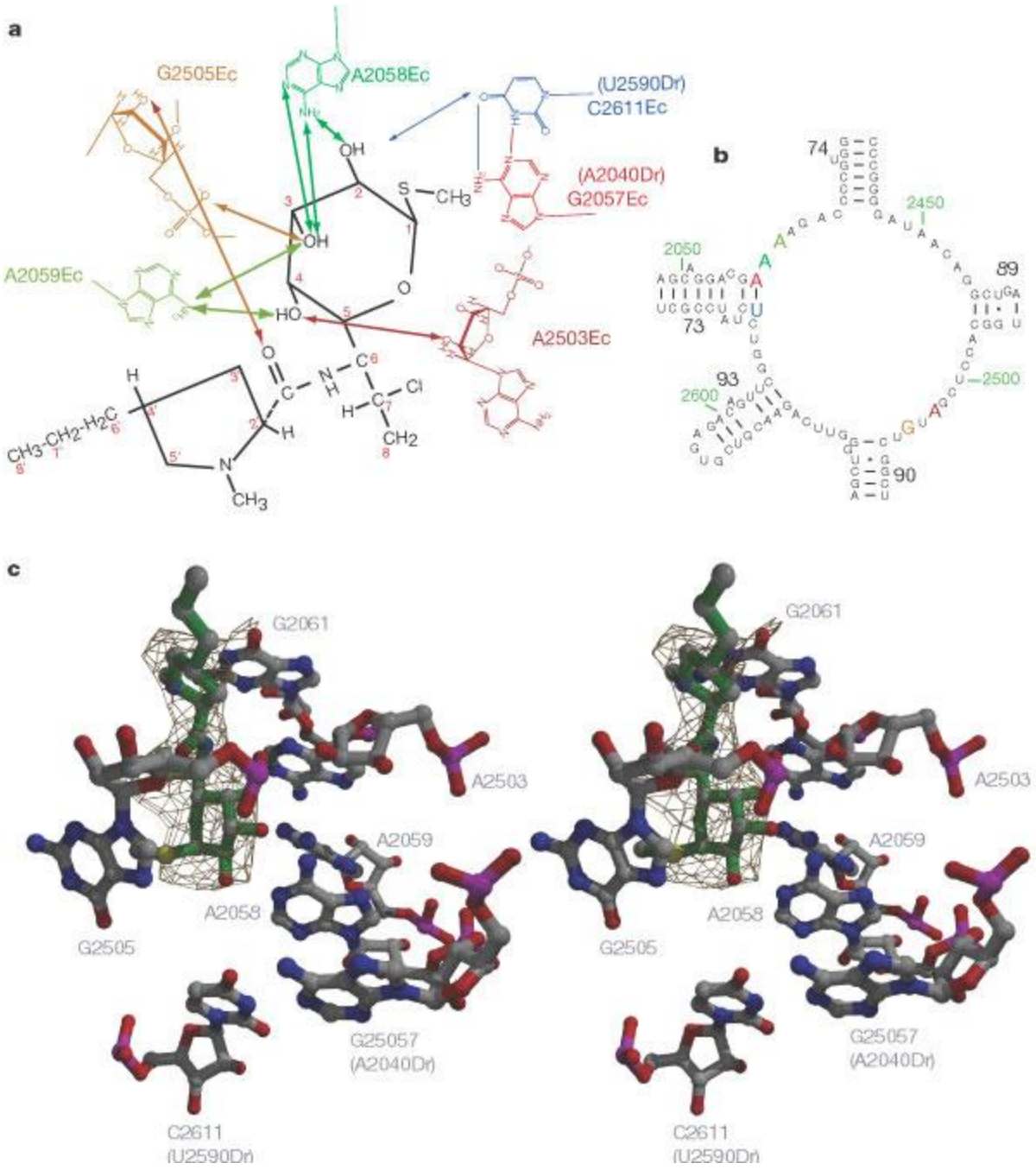


Figure 11. Interaction of chloramphenicol with the peptidyl transferase cavity. A) Chemical structure diagram of chloramphenicol showing the interactions (arrows) of its reactive groups with the nucleotides of the peptidyl transferase cavity. B) Secondary structure of the peptidyl transferase ring of D. radiodurans showing the nucleotides involved in the interaction with chloramphenicol (coloured nucleotides). C) Stereo view showing the nucleotides interacting with chloramphenicol at the peptidyl transferase cavity of D. radiodurans. The antibiotic is shown in green. Nucleotide numbering is according to the E. coli sequence. Putative Mg ions (Mg-C1, Mg-C2) are indicated. Adapted with permission from [49]

1.2.2.4 Macrolides

Macrolides (Fig. 12) are large macrocyclic lactone rings with one or more pendant glycosidic residues, commonly cladinose and desosamine, and the ring size ranges from 14- to 16-membered. Most macrolides are semi-synthetic derivatives of erythromycin, a macrolide which was first isolated from soil bacteria in 1949[50]. Compounds such as clarithromycin, a 14-membered ring with cladinose and desosamine decorations, and azithromycin, a 15-membered ring with an inserted nitrogen, show activity against Gram-positive bacteria, with limited usage against Gram-negative bacteria. Because macrolides naturally accumulate in the lungs, they are commonly used to treat respiratory infections. As all currently used macrolides are derivatives of erythromycin, there are limited possibilities for future development. A method starting with basic building blocks of macrolides has been proposed as a highly modular, fully synthetic alternative route[50]. Ketolides, such as telithromycin, are macrolides in which the C3 cladinose of erythromycin has been replaced with a ketone. In the case of telithromycin, the compound also has an alkyl-aryl arm that extends off a carbamate fused to the main ring structure. These compounds bind in the same location as the other macrolides, but show increased acid resistance thanks to the lack of the labile cladinose group[51]. Macrolides are translation inhibitors, binding to the 50S subunit within the ribosomal exit tunnel, a cavity which extends from the peptide transfer center through the 50S subunit, through which the nascent peptide extrudes from the ribosome into the cytosol (Fig. 13). The binding site is just before a natural constriction point created by ribosomal proteins L4 and L22 jutting into the exit tunnel. Macrolides block off this tunnel, preventing peptide extrusion, stalling the ribosome and thereby halting translation. Ketolides, lacking the bulky cladinose ring, allow for a longer peptide segment to be formed before translation is halted[52]. Beyond a simple steric obstruction, it has been indicated that the stalling of the ribosome occurs in a context-specific

manner, that is, specific sequences and amino acids result in stalling due to interactions of the macrolide within the tunnel causing upstream effects at the peptidyl transferase center[53]. One common mode of resistance to macrolides is modification to the 23S rRNA, specifically the alteration of A2058, which plays a key role in the binding of macrolides. Resistance genes code for proteins which methylate this position, leading to a loss of binding affinity and decreased sensitivity to the compound. Other methylations, such as at G748 also lead to resistance[54]. Drug efflux is another common mode of resistance.

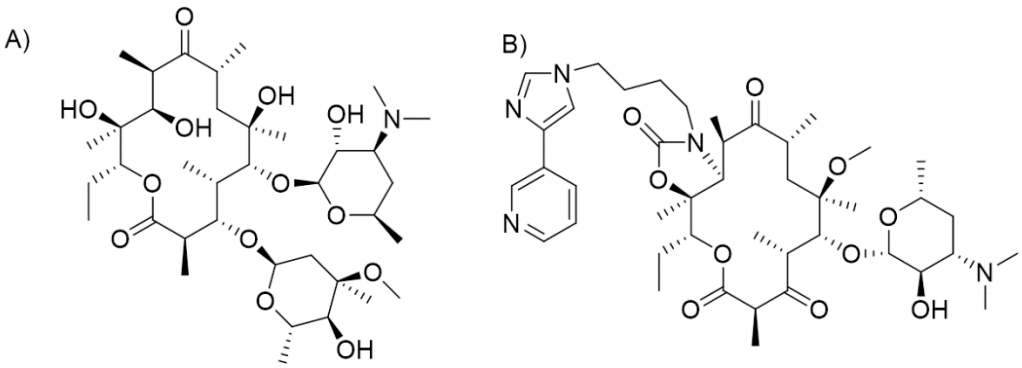


Figure 12. Structure of A) erythromycin and B) telithromycin

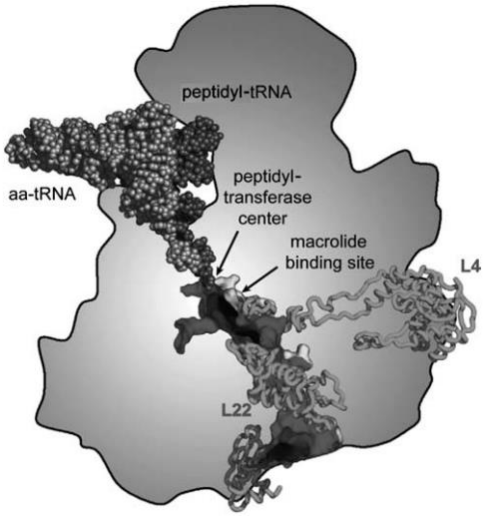


Figure 13. Schematic of ribosome with macrolide binding site indicated. Adapted from [55]

1.2.2.5 Streptogramins

Streptogramins are macrocyclic lactones subdivided into two classes: A and B. The two classes work synergistically, as they are bacteriostatic when used alone but bactericidal when used together, and so are used as mixtures, as with quinupristin/dalfopristin (Fig. 14). The streptogramins are most effective against Gram-positive bacteria and are generally used to treat cases of vancomycin resistant *Staphylococcus* and *Enterococcus*. Class A streptogramins have macrolide-like structures and generally have a high degree of unsaturation, whereas class B streptogramins are cyclic depsipeptides[56]. Streptogramins, like macrolides, halt translation and even have a similar binding site. Streptogramin A blocks substrate access to the acceptor and donor sites of the PTC, while streptogramin B prevents peptide bond synthesis and induces the detachment of incomplete protein strands and blocks the exit tunnel similarly to macrolides[56]. The synergy of the two classes stems from conformational changes to the 23S rRNA which occur upon streptogramin A binding which increase affinity for streptogramin B[57]. Resistance is acquired in a similar manner to macrolides: the chemical modification of the 23S rRNA leads to decreased binding efficiency[57].

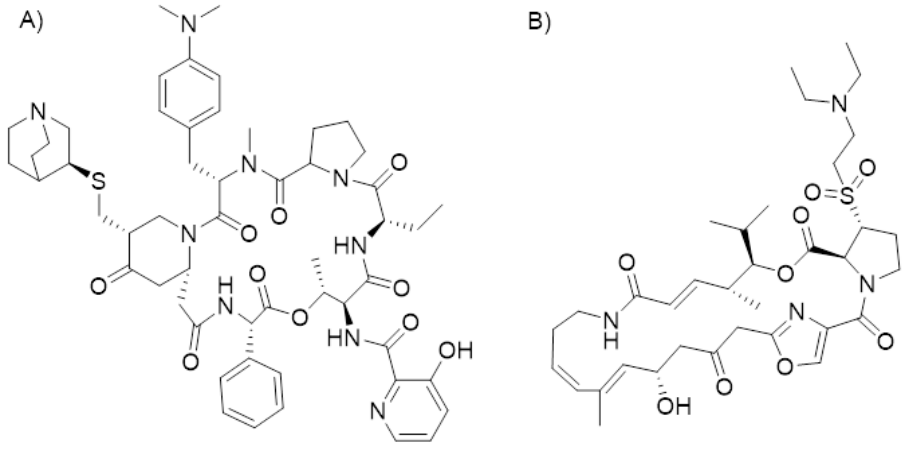


Figure 14. Structure of A) quinupristin, a streptogramin B, and B) dalfopristin, a streptogramin A

1.2.2.6 Oxazolidinones

The oxazolidinones are a relatively new (clinically approved in 2000) antibiotic which are highly active against Gram-positive bacteria but show limited activity against Gram-negative bacteria. Linezolid (Fig. 15) is the first of a small number of currently available oxazolidinones; its primary uses are against penicillin resistant *S. pneumoniae,* endocarditis, vancomycin-resistant strains, and tuberculosis[58]. Although this compound shows excellent bioavailability, it does present some unwanted side effects, such as gastrointestinal disturbance. Oxazolidinones bind to the 50S subunit of the ribosome. Linezolid competes with chloramphenicol and lincomycin for binding, indicating that they have close binding sites, but does not inhibit peptidyl transfer as the other two. Oxazolidinones inhibit the binding of fMet-tRNA to the P-site and inhibits the formation of the 70S ribosomal complex. In ribosomes that have already formed the 70S, oxazolidinones prevent translocation of the peptidyl-tRNA from the A-site to the P-site[59]. The only resistance mechanism described for linezolid thus far is target modification. The most common mutation is a change of 23S G2576 to U2576, but other modifications conferring resistance have been found at position 2032 and 2447 in the 23S of *E. coli*[58].

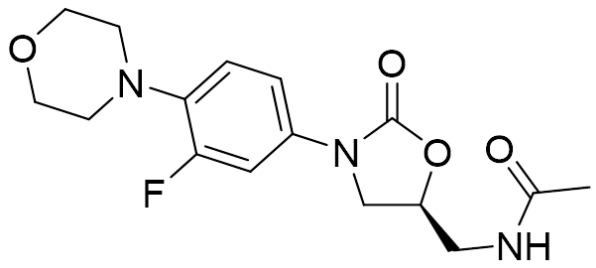


Figure 15. Structure of linezolid

1.2.2.7 Lincosamides

Lincosamides (Fig. 16) are small antibiotics containing a mycarose sugar which, although structurally distinct from macrolides, nevertheless operate in a similar fashion and share common binding sites and modes of resistance. The lincosamides, unlike macrolides and streptogramin B, directly interfere with the peptidyl transfer reaction[60]. Lincosamides are primarily used to treat infections from anaerobic bacteria and have broad-spectrum coverage. Clindamycin is one clinically available lincosamide, which is commonly used in dentistry[61] and can also be used to treat some cases of MRSA[62]. Clindamycin can also be used to great effect as an antimalarial when used in combination with chloroquine or quinine[63], but its clinical use as an antibacterial is marred by several side effects as well as an increased risk in *Clostridium difficile* colitis in hospital patients[64].

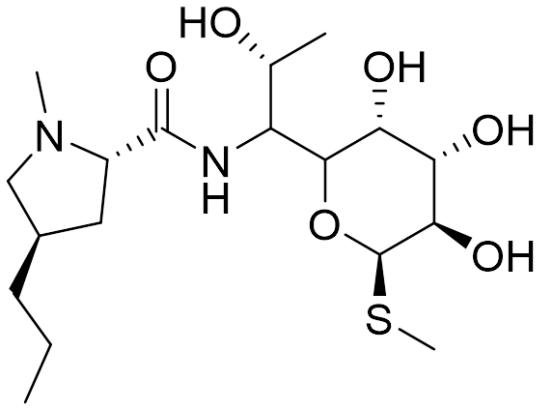


Figure 16. Structure of lincomycin, a lincosamide

1.2.3 Antibiotics which have other internal targets

Some antibiotic classes have targets within the bacterial cell which are not on the ribosome, such as DNA proteins.

1.2.3.1 Quinolones

Quinolones (Fig. 17) are broad-spectrum antibiotics based on a 4-quinolone core. The first discovered compound was nalidixic acid in 1962, which is technically a napthyridine due to the extra nitrogen in its ring structure. Other derivatives were subsequently developed and put to use clinically, but the compounds suffered from poor bioavailability and relatively low activity[65]. The quinolone family received renewed interest with the development of a fluorinated derivative, flumequine. Although the use of this compound was discontinued due to ocular toxicity, the production of quinolones moved strictly toward fluorinated derivatives, creating the subclass of fluoroquinolones[66]. These fluorinated derivatives showed superior bioavailability and effectivity, with ciprofloxacin currently the most potent quinolone available on the market, used primarily in the treatment of Gram-negative infections[65]. Quinolones target two key type II topoisomerases, DNA gyrase and DNA topoisomerase IV (Topo IV). The former protein is responsible for introducing negative supercoils into bacterial DNA, an aspect of crucial importance to bacterial replication which is not present in eukaryotic cells, making it an ideal target.[61] The latter protein is a decatenating enzyme which is responsible for unlinking sister chromosomes during replication and for relaxing positive supercoils in DNA[67]. Quinolones bind to the gyrase/topoisomerase-DNA complex, blocking DNA synthesis and halting cell division[68]. There are several major modes of resistance to quinolones. First, chromosomal-mediated resistance stemming from mutations in the *gyr* genes can lead to decreased binding and low-level resistance. Additionally, effective drug concentration can be reduced either through decreased permeability or improved drug efflux[68].

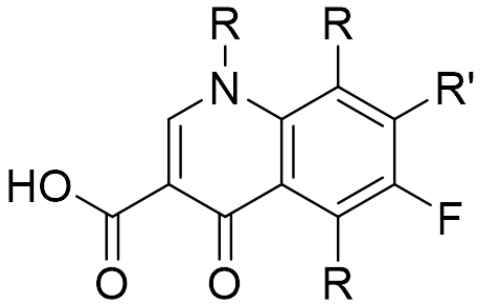


Figure 17.General structure of quinolones. The R' group is usually piperazine, the presence of fluorine makes the compound a fluoroquinolone

1.2.3.2 – Sulfa drugs

Sulfa drugs, or sulfonamides (Fig. 18), were technically the first antibiotics, developed in the early 1930's by Gerhard Domagk[69]. These compounds inhibit the production of folic acid by targeting dihyropteroate synthase. Although the use of sulfonamides quickly spread, they were blighted by high incidence of allergic reactions, which could be extremely severe in some cases, and by widespread resistance[70]. They are still sometimes used in combination with inhibitors of dihydrofolate reductase, a protein further down the synthetic pathway to folic acid, such as trimethiprim.

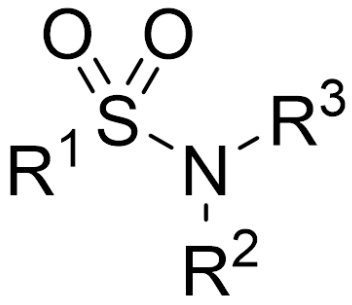


Figure 18. General sulfonamide structure

CHAPTER 2. ASSAYS AND TECHNIQUES

In order to discover antibiotics, characterize their structure, and elucidate their mechanisms, a wide variety of assays and chemical techniques are utilized. This chapter will examine many of the tools which scientists use to discover, identify, and characterize antibiotics. This chapter is not concerned with organic synthesis or the precepts of synthesizing synthetic or semi-synthetic antibiotics. We will begin with how advances in screening allow for the identification of potential antibiotic candidates, then examine various visual and analytical techniques and how they have aided in the discovery and development of currently used antibiotics.

2.1 Compound screening

The first step in the development of a new antibiotic is the identification of a potential compound which displays antibacterial activity. As there are countless organisms which produce diverse compounds, as well as synthetic compounds developed for other purposes, it is necessary to utilize efficient screening methods to test a vast number of potential candidates rapidly. In the 1940s, Alexander Waksman developed a method to screen compounds from soil-derived *Streptomyces* samples by looking for zones of growth inhibition on an overlay plate, similar to the method by which Fleming accidentally discovered penicillin[71]. Waksman's method led to the discovery of streptomycin, discussed earlier. The "Waksman Platform" was responsible for the discovery of most of the major antibiotic classes during the Golden Era. Since then, screening methods have evolved as the number of compounds isolated from natural sources has skyrocketed[72]. Currently, compound screening can be done rapidly and efficiently in an automated fashion. Advances in engineering and robotics have led to high-throughput instruments capable of assaying hundreds if not thousands of compounds a day[73]. Newer machines are even capable of ultra-high throughput,

processing hundreds of thousands of compounds a day[74]. Recently, an interesting technology for screening uncultivable microbes, which comprise the vast majority of microbial populations, based on genetic data, but cannot be grown under standard laboratory conditions. Taking advantage of advances in microfluidics, Nichols et al. was able to create an isolation chip (dubbed iChip) which allowed for culture separation and incubation from a given water or soil sample[75]. Screening assays can be based on various methods of detection, including radiochemical, fluorescence and luminescence.

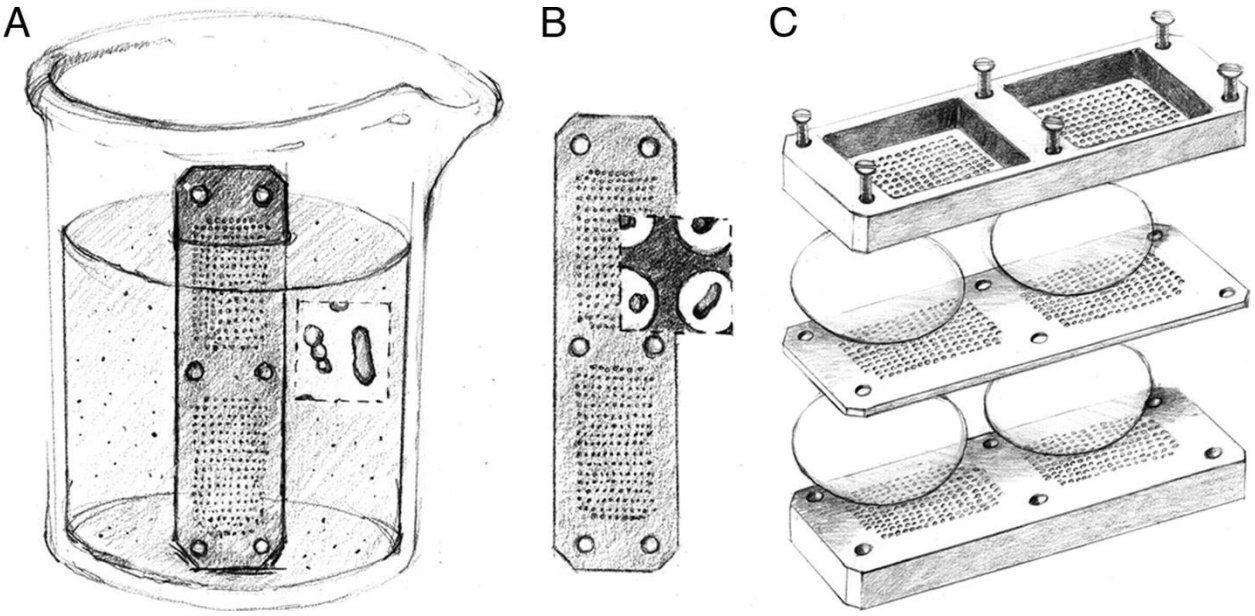


Figure 19. The iChip (a) consists of a central plate (b) which houses growing microorganisms, semi-permeable membranes on each side of the plate, which separate the plate from the environment, and two supporting side panels (c). When the central plate is dipped into a cell suspension in molten agar, the through-holes capture small volumes of the suspension which solidify as plugs. The membranes are attached and the iChip is placed in a soil sample. Adapted with permission from [76]

Radiochemical assays are highly precise, for example, *in vitro* receptor assays can detect binding constants ($K_{d)}$ in the pM range, and can detect protein-protein interactions at the nM range[76]. Radioisotopically-labeled compound uptake can also be monitored precisely. This method suffers drawbacks from high reagent cost, and subsequently high assay cost, as well as difficulty in miniaturization[74]. Radiolabeling is discussed in greater detail in a later section.

Fluorescent assays are diverse, cost-effective, and have low detection limits. There are many fluorescent detection methods, such as fluorescent anisotropy (FA), which uses molecular rotation to measure bimolecular association events[77]. A common fluorescent method is fluorescence energy resonance transfer, or FRET. FRET is based on energy transfer between a fluorophore donor and a chromophore acceptor which is distance-dependent. The fluorescent emission of the acceptor can be resolved in a time-dependent fashion. FRET can be used for ligand-binding, enzyme turnover, and even whole-cell assays[76].

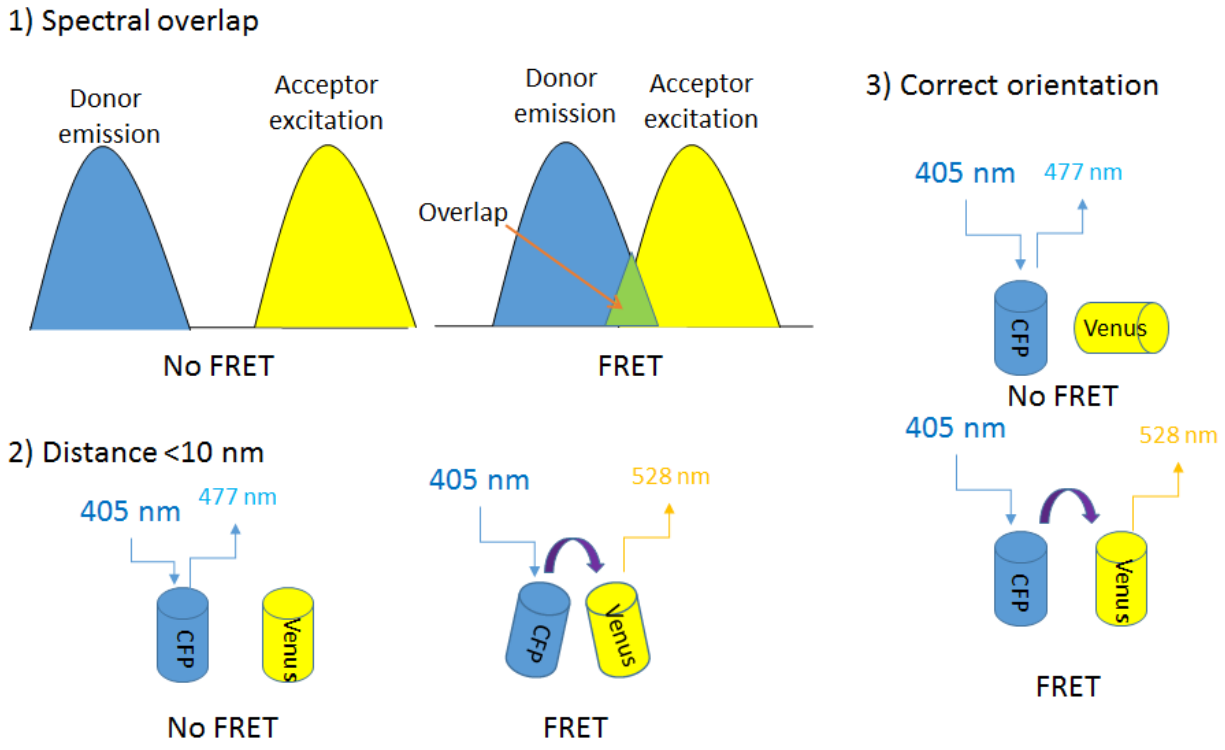


Figure 20. Conditions required for FRET. Adapted from [78]

Chemiluminescence (CL) assays are similar to fluorescence assays and the equipment used in these assays are often capable of measuring luminescence as well as fluorescence. CL predominately takes advantage of luciferase reporter genes in cell based assays and alkaline phosphatase/horseradish peroxidase in high-sensitivity enzyme-linked immunoabsorbent assays

(ELISA). In fact, CL is most commonly applied in immunoassay-based detection[74]. The use of a chemiluminescence in an assay is clearly laid out by examining the ELISA assay in more detail. In direct ELISA, an antibody to a particular antigen is linked to a reporting enzyme. This conjugated antibody is then added to a sample containing the antigen to the antibody. The substrate for the enzyme is added, allowing for a measurable change in the solution of color or light[77]. If luciferase is linked to the antibody, luciferin can be used as a substrate and the subsequent luminescence measured and quantified. There are also indirect and 'sandwich' methods for ELISA which have additional steps, for instance an indirect ELISA will use an unlabeled primary antibody which binds the antigen and a labeled secondary antibody which binds the primary antibody[79].

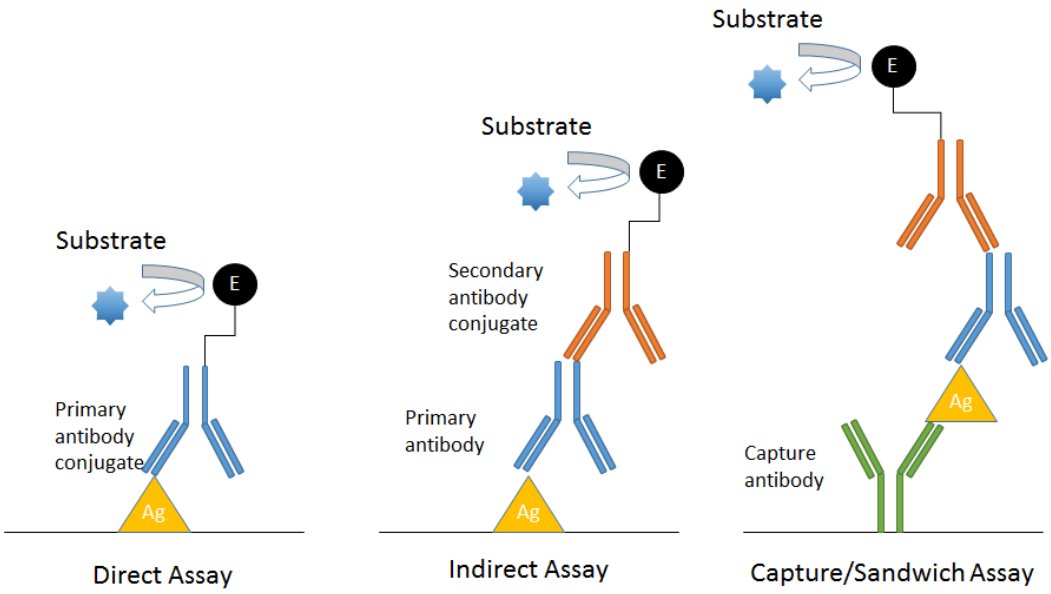


Figure 21. ELISA methods. Ag = antigen, E = enzyme

Indirect ELISA has several advantages: multiple secondary antibodies can bind the primary antibody, giving a stronger signal, and different primary antibodies can use a single secondary antibody.

Recent advances in genetics have had an astounding impact on natural product discovery. As more and more genetic information becomes decrypted and catalogued, the field of metagenomics has risen to profoundly shape the way in which researchers approach natural products. As techniques to garner genetic information improve, the libraries of genomic information expand. Sequencing is often done via the Sanger method, which utilizes fluorescently labeled dideoxynucleotides to arrest DNA synthesis, leaving a truncated molecule. Through many rounds of synthesis, fragments of every conceivable length are created, which are then separated by size electrophoretically, with the fluorescent tag indicating which nucleotide is at the end of the sequence. In this manner, a Sanger 'trace' is created which is converted to a DNA sequence[80].

Although Sanger sequencing is the traditional route, newer generations of sequencing technology have utilized different methodologies. 454 sequencing, for example, utilizes a luciferase luminescence based assay to determine the base being added to the sequence in real time, however this technology struggles with homopolymeric sequences (multiples of the same nucleotide in sequence)[80].

Using sequence analysis, genes which encode biosynthetic pathways can be parsed out and many novel compounds have been discovered in the genomes of microbes which cannot be cultivated using normal methods[81]. Recently, a group developed a technique to selectively retrieve compounds from a crude cell extract in a reaction-dependent manner, allowing them to extract compounds which contained specific moieties common to antibiotics, such as β-lactams. They coupled their probes to information obtained from genome mining and were able to isolate a compound which was anticipated from sequencing but had previously not been obtained as a pure isolate[82].

2.2 Whole cell and blotting assays

2.2.1 Blotting to assess compound target

Blotting is so called because it involves the transfer of a substance from a gel to a different surface for probing. A blotting technique to detect specific DNA fragments was developed by Edwin Southern and received his name, the Southern blot. In reference to this assay, subsequent assays developed to assess RNA, protein, and post-translational modifications were termed Northern, Western, and Eastern blots respectively. Hybrids of these assays, such as Southwestern blots, are also utilized but will not be discussed here. These blots are of great merit in discerning the specific effects of a compound on a bacterial target, with these assays being able to assess a majority of possible targets. The Southern blot, as previously noted, detects specific DNA fragments, which are separated electrophoretically, using probe hybridization. DNA fragments are run through an agarose gel, after which they are transferred to a nitrocellulose sheet and treated with a probe. The probe can be made from either DNA or RNA, and is complementary to the fragment it binds to. The probe is tagged either with a radioactive isotope or with a fluorescent or chromophoric label[83]. This technique is used to identify Human Papilloma Virus infections clinically, although using polymerase-chain reaction (PCR) has become more common[84]. The Northern blot is used to measure gene expression through the presence of RNA. An RNA extract is run through an agarose gel, which often contains formaldehyde to limit secondary structure, after which the RNA is transferred to a nylon membrane and visualized with hybridization probes of either DNA or RNA[85]. The probes are labelled either radioactively or else function through chemiluminescence. The chemiluminescent probes can either be attached to the enzyme (such as luciferase) or a substrate for a ligand which is attached to the enzyme, for example, a biotin-labelled probe and an avidin-labelled enzyme[86]. Northern blots are commonly used in cancer

research to observe the changes in expression levels that occur in tumor cells[84]. A large database of Northern blots, BlotBase, has been established and contains over 700 published blots[87]. The Western blot is used for protein, and utilizes an antibody probe for visualization. The proteins can be separated on a gel based on many factors, including isoelectric point, molecular weight, and charge. Once the gel has been run, the proteins are transferred to a membrane, usually nitrocellulose, and treated with a primary antibody which binds that protein[88]. Typically, the primary antibody is visualized with a secondary antibody which is specific to the primary antibody, although single-step visualization methods have been developed[89]. Primary antibodies for a vast array of proteins are available commercially. SDS-Page is a common type of western blot which uses sodium dodecyl sulfate (SDS) to denature proteins before they are run on a polyacrylamide gel. After visualization, a desired protein band can be cut from the gel and used in further experiments. The Eastern blot is the least common of the directional blots, and is used to visualize post-translational modifications, such as lipids and glycoconjugates, on proteins[90]. Eastern blotting suffers from a somewhat indistinct definition, being viewed mainly as an extension of Western blotting, as the initial gel electrophoresis and membrane transfer are essentially identical, and the definition and purpose of an Eastern blot has varied over the years[91-93].

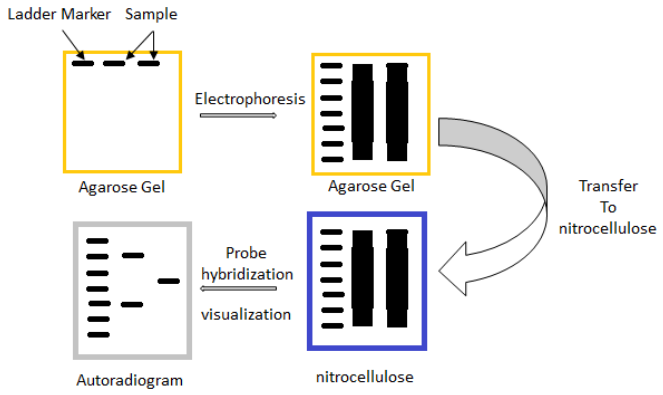


Figure 22. Southern blot method. Other blots follow the same general procedure with minor variations depending on which blot is being used

2.2.2 Minimum inhibitory concentration (MIC)

For any given compound, its general effectivity against a given strain can be assessed using an MIC assay. There are four main methods of testing the MIC: Disk diffusion, agar dilution, broth dilution and broth microdilution.

Disk diffusion is a quick and inexpensive method of testing strains against antibiotics, but suffers from a relative lack of accuracy compared to the other methods. In this method, an agar plate is seeded with an inoculum of known dilution, and an antibiotic is introduced via strip or wafer. The zone of inhibition generated by the antibiotic is measured[94]. The zone size is susceptible to a number of factors, making some disk diffusion methods only semi-quantitative[95].

In the agar dilution method, agar plates are prepared with an incorporated antibiotic agent with a range of concentrations. An inoculum is seeded on the plates and the MIC is determined as the lowest concentration plate which exhibits no microbial growth. One of the main problems with this method is the interpretation of what qualifies as the first plate with no bacterial growth[95].

The broth dilution method is identical in both macro and micro considerations, the difference being the volume of liquid. In the macrodilution method, tubes contain several mL of liquid while in the microdilution method, 96-well plates which hold a final volume of 100 µL are used. This makes the microdilution method much more economical and practical[83]. In this method, a 96-well plate is seeded with an inoculum, after which the compound of interest is added to the top row of wells, which contains a double volume of inoculum. The excess liquid is transferred sequentially down the rows, resulting in a 2-fold dilution of the compound with each subsequent row. In this manner, a total of 8 concentrations can be tested on a single plate. The absorbance of the liquid at 600nm

is taken before and after an overnight incubation and the resultant data can be converted to an MIC[96].

2.3 Cell fraction/lysate assays

Many of the antibiotics discussed in the first section target the ribosome. Cell-free assays are available for both prokaryotic and eukaryotic systems which consist of a cell extract containing intact ribosomes. To the extract amino acids and a luciferase plasmid are added. When testing a compound, inhibition of translation prevents the production of luciferase, leading to a lack of luminescence when luciferin is added[97]. In short, dimmer wells in the plate correspond to greater inhibition of translation by a given compound. With this assay, the inhibitory concentration at which fifty percent of translation is abated, the IC_{50}, can be determined.

2.3.1 RNA Footprinting

RNA footprinting is a method of identifying interactions of RNA with proteins and ligands. In essence, RNA footprinting is a protection assay wherein digestion or chemical modification of RNA is locally inhibited through ligand binding or inherent RNA structure[98]. This provides reliable data on interactions within the RNA, although it cannot in itself distinguish between direct protection and indirectly induced protection caused by conformational changes due to interactions at different locations. There are various probes available for footprinting experiments and can either be sequence or structure specific. An example of a sequence-specific probe, as well as one of the more versatile, is dimethyl sulfate (DMS). DMS methylates the Watson-Crick face of adenine and cytosine and the Hoogsteen face of guanine[99]. Structure-specific probes, such as N-methylisatoic anhydride (NMIA) form adducts with the 2’ oxygen regardless of base identity. This chemistry is utilized in RNA selective 2’-hydroxyl acylation analyzed by primer extension

(SHAPE) assays. SHAPE takes advantage of the adducts formed to analyze RNA folding[100]. The RNA fragments generated during a footprinting experiment is visualized using radioactive labels, most often ^{32}P, and the experiment is highly susceptible to degradation from RNases so care must be taken to avoid contamination during the experiment[101]. Our lab previously used footprinting to observe peptide interactions in the ribosomal exit tunnel, using macrolide-peptide conjugates[102]. A similar technique can be done with DNA to investigate sequence-specificity of DNA binding proteins[103].

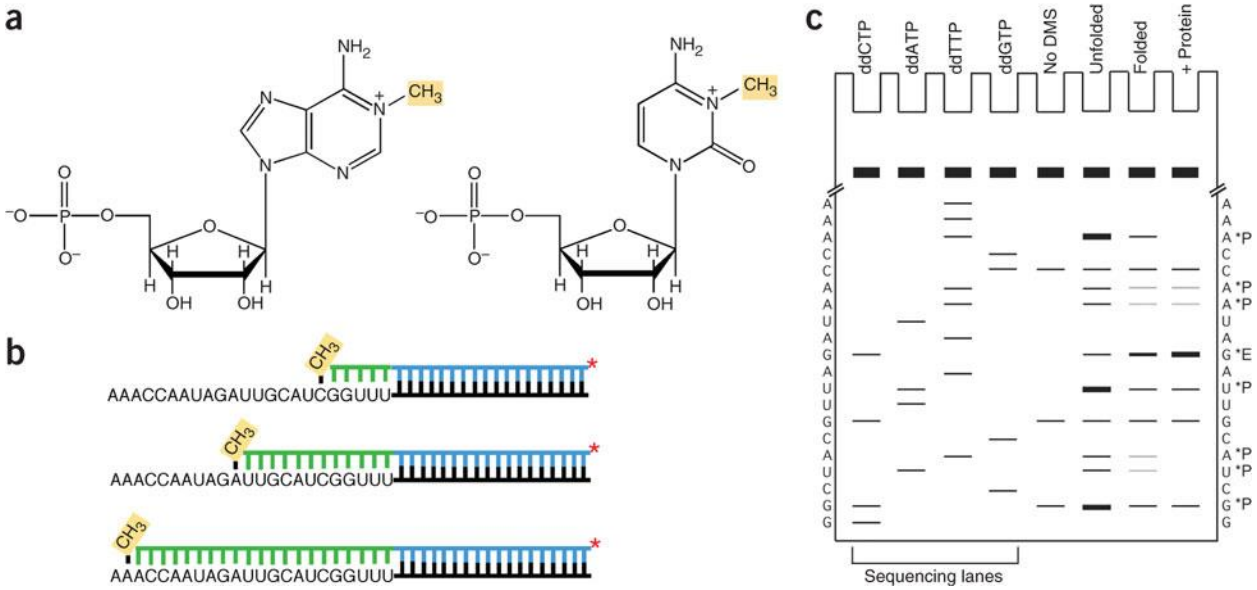


*Figure 23. RNA footprinting. **a**) The modifications detected by this method are methylation at N1 of adenine and N3 of cytosine, as indicated in yellow. (**b**) Primer extension. The DMS modification reaction is carried out under limiting conditions so that each molecule has no more than an average of one detectable modification. Reverse transcription is illustrated (product in green) proceeding from a radiolabeled primer (blue line) until it is blocked at a position 1 nt upstream from a methylated A or C nucleotide. (**c**) PAGE analysis of reverse transcription products. Sequencing lanes at the left are used to determine the position of modification for each experimental band. Adapted with permission from [101]*

2.3.2 Affinity Chromatography

Affinity chromatography is a method of extracting a desired compound from a crude extract using an immobilized linker which will pull out the desired compound and allow the unwanted remains

to pass through the column. This technique is most commonly used to purify proteins, but can also be used for other compounds, such as DNA and RNA[104].

One method of affinity chromatography is called immobilized-metal affinity chromatography, or IMAC. IMAC is based on the natural affinity of transition metal ions such as Zn^{2+} and Ni^{2+} for histidine and cysteine[105]. Columns with immobilized metal ions have been utilized for decades to purify recombinant proteins by adding a series of histidines, a His-tag, which allows the recombinant protein to remain in the column during elution[106]. By immobilizing D-Ala-D-Ala moieties, discussed in chapter 1, affinity chromatography has been used for the discovery and purification of glycopeptide antibiotics[107].

More recently, IMAC has been utilized to purify membrane proteins. Although membrane proteins constitute approximately 30% of the human proteome, very little is known about membrane proteins compared to soluble proteins, which is reflected by their underrepresentation on structure databases such as the Protein Databank (PDB). A standardized IMAC procedure for purifying membrane proteins is available and has been made simpler through the development and optimization of detergents[108]. Additionally, IMAC is also useful in purifying proteins which contain the zinc-finger motif, an important DNA binding motif[109].

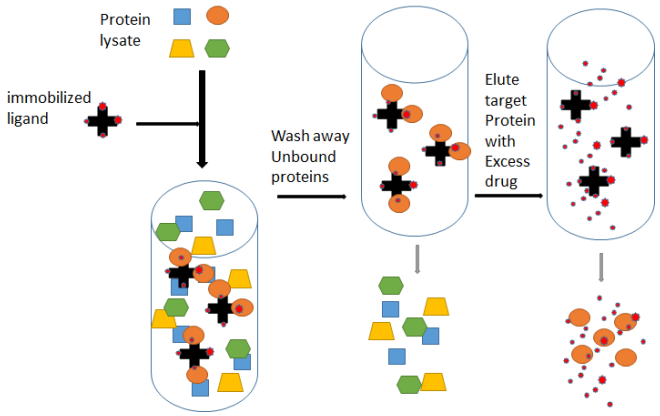


Figure 24. General diagram of affinity chromatography

2.3.3 Thermal Shift

Once a protein has been isolated, it is possible to verify the binding of a ligand using a thermal shift assay. This assay, first described in 1958, operates on the principle that the binding of a low molecular weight ligand often increases the thermal stability of a protein, effectively raising its melting point[110]. Circular dichroism and differential scanning calorimetry are common, inexpensive methods but they suffer from low throughput and require relatively large amounts of protein. Newer methods have been developed, the first being the thermofluor assay[111].

The thermofluor assay, first described in 1991, uses a dye which binds nonspecifically to a protein and is displaced by water as the protein unfolds, causing an increase in fluorescence [112]. This method is not particularly well suited for measuring protein-protein interactions and protein aggregation can interfere with fluorescence. Variations of this assay have been developed with nucleophile-specific[113] and rigidity-sensitive dyes[114], as well as utilizing intrinsic tryptophan fluorescence[115].

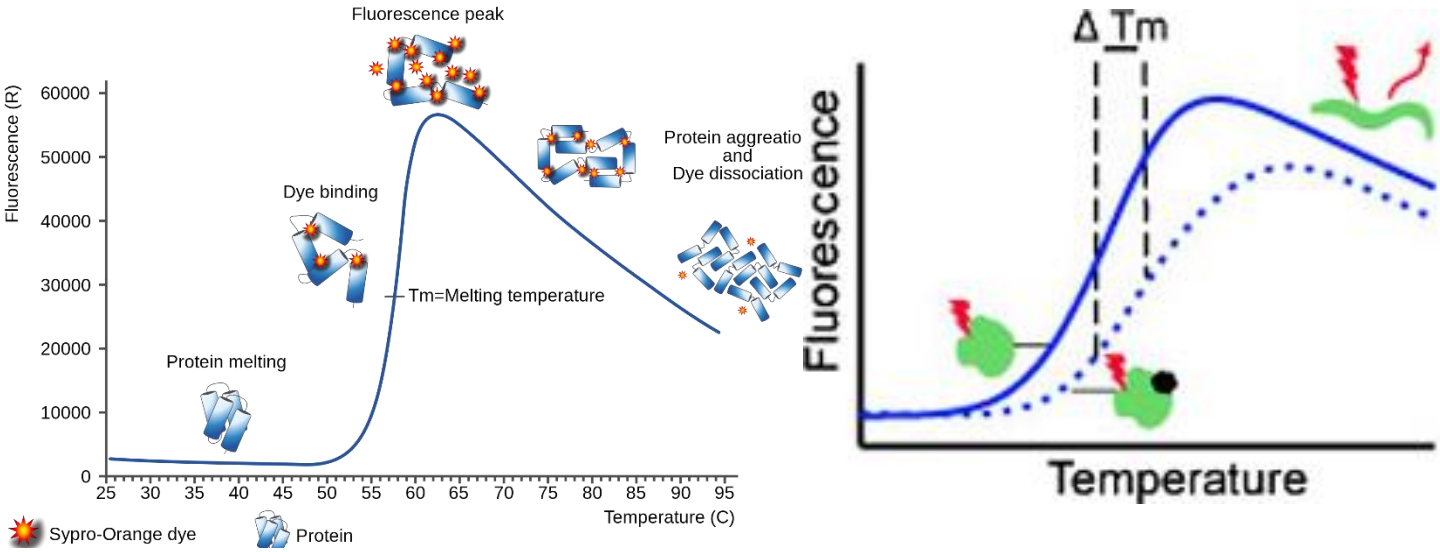


Figure 25. Thermal shift assays. A) The principle of Tm detection with fluorescence and B) the difference in Tm associated with ligand binding. Adapted with permission from [113]

2.3.4 Radiolabeling

Radiolabeling compounds is a technique that allows for many assays, such as binding affinity and saturation. When synthesizing a radiolabeled compound, many factors must be taken into consideration, including the half-life of the isotope – which may make experiments time-sensitive – and the type of radiation emitted by a given isotope, which may limit the detection methods available.

A saturation analysis using a radioligand can yield the receptor affinity and can be done through two methods. First, the amount of radioligand added can be increased while maintaining a constant specific activity. Second, a constant concentration of radioligand can be used and the specific activity decreased by the addition of unlabeled ligand[116]. A Scatchard plot can be used to find the binding constant of the radioligand, K_d.

Once K_d for a radioligand is found for a target receptor, it can be used in competition binding assays to determine the binding affinities of other, unlabeled compounds. A wide range is usually tested, against a fixed concentration of the labeled ligand[116]. Radiolabeling was utilized to determine that lipopeptides are membrane active[117].

Radiolabeling is hampered by the high costs of isotopes, the intrinsic chemical hazard of working with radioactive materials, and the stringent waste disposal requirements.

2.4 Imaging

Cell imaging is a powerful resource for visualizing cellular structures, tracking proteins, and more. Although the inventor of the microscope is difficult to identify exactly, light microscopy garnered

attention in the scientific community thanks to the insights into microscopic life it provided. Robert Hooke's *Micrographia* and the work of Antonie van Leeuwenhoek, who used his 300x magnification microscope to discover red blood cells and spermatozoa - and later microorganisms - were instrumental in establishing the utility of the light microscope for biological studies[118, 119]. Since these early years of development in the late 1600s, microscopy has evolved beyond simple optics. The advance from light microscopy to electron microscopy and x-ray techniques led from being able to see individual cells to being able to see individual atoms, bringing in a host of marvelous discoveries, including important information on antibiotic structure and function.

2.4.1 Light microscopy

Optical microscopy has been fundamental in both the biological and medical fields for centuries. There are four main modes of a typical light microscope: Bright field, where contrast comes from light absorbance, cross-polarized illumination, where contrast comes from the rotation of polarized light through the sample, dark field, where contrast comes from light scattering by the sample, and phase contrast, where contrast comes from the interference of different path lengths through the sample. Optical microscopy is ultimately limited by the wavelengths available in visible light and diffractions, resulting in a max resolution of 200nm, although developments in lenses and techniques have allowed the resolution limit to be surpassed[120].

Fluorescence microscopy is an offshoot of optical microscopy, utilizing lasers to excite fluorescent dyes and tags. A particularly important discovery in this field was the isolation of green fluorescent protein (GFP). Discovered and isolated from *Aequorea* jellyfish, GFP revolutionized fluorescent microscopy by making it possible to use GFP recombinately to tag proteins and structures of interest, allowing them to be visualized[121, 122].

Confocal microscopy is a powerful type of fluorescence spectroscopy. In confocal microscopy, illumination is confined to a diffraction-limited spot in the sample and detection is confined via an aperture in front of the detector, which results in an optical sectioning effect[123]. This technique not only provides excellent resolution but also allows for pseudo 3-dimensional images to be constructed by imaging sample sections from top to bottom. Advances in fluorescence microscopy have also enabled multiphoton microscopy (MPM). MPM uses nonlinear processes, most commonly two-photon excitation (2PE), although techniques such as three-photon excitation and second harmonic generation (SHG) are also utilized[124]. One major advantage of 2PE is tissue penetration, as the use of multiple low-energy photons scatter less and a signal is generated only at the focal point. Harmonic generation occurs when multiple photons interact with non-symmetrical structures without being absorbed, the information divulged by this scattering has been used to image membranes and plant structures[125].

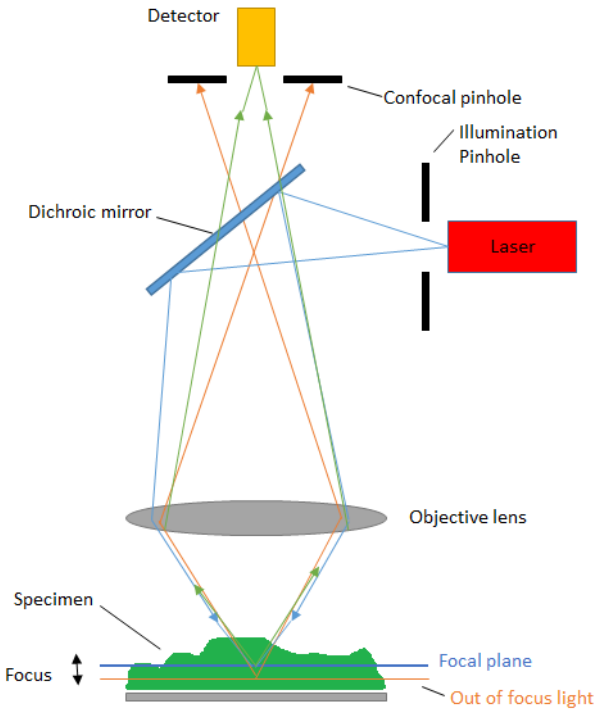


Figure 26. Schematic of a confocal microscope

2.4.2 Electron microscopy

Electron microscopy overcomes the limitations of light microscopy by using electrons as a source of illumination. The significantly shorter wavelength of electrons allows for resolutions up to and even beyond 50 picometers[126]. The two main modes of electron microscopy are transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

TEM is an electron approximation of standard optical microscopy: an electron beam is fired at a sample, the occlusion and absorption of electrons by the sample generates a bright field image[127]. Samples must be thin enough to allow the passage of electrons, typically 60-100 nm thick, and additionally biological samples must be stained with heavy metals to provide contrast[128]. TEM has been used to visualize the effect of tamoxifen, an anticancer drug, on bacterial cell walls[129]. SEM relies on energy emissions and backscattering of electrons to form a map of a sample's surface. Although the technique does not offer the same resolution as TEM, SEM is capable of handling much larger samples and has excellent depth-of-field, making it useful for visualizing the three-dimensional shape of the sample[130]. Samples are generally sputter coated with a thin layer of gold, palladium, or other heavy metals to create a conductive layer which allows for imaging, although uncoated samples can be imaged[131].

Scanning tunneling microscopy (STM) is a method used to image surfaces at the atomic level. This technique utilizes a metal tip which ends in a single atom to probe the surfaces of materials using the principles of quantum tunneling[132]. Atomic force microscopy is a variant of the scanning tunneling microscope, using a cantilever with a tip ending in a single atom which passes over the surface of a material. As the cantilever interacts with the surface, it is displaced, this movement is measured to yield information about the material surface[133]. This technique has

allowed for chemical structure determination and even the study of single molecule recognition events[134].

2.4.3 X-ray crystallography

One of the most powerful tools available to the biochemist is X-ray crystallography. By observing diffraction patterns from a crystal of a given substance, an electron density map can be derived. From this electron density map, the positions of atoms, their chemical bonds, and other information can be determined.

The first structure of an organic compound, hexamethylentetramine, was solved in 1923[135]. This was followed by structures of fatty acids, which led to more complex structures, such as phthalocyanine in the 1930s[136]. Dorothy Hodgkin won the Nobel prize in chemistry in 1964 for solving the structures of cholesterol, penicillin, and vitamin B12, and she later solved the structure of insulin[137].

The first protein to be solved was sperm whale myoglobin in the 1950s by John Kendrew, for which he won the 1962 Nobel Prize[138]. Since that success, over eighty-thousand protein crystal structures have been solved and can be accessed on the PDB. One of the more recent breakthroughs in crystallography is the solving of the whole bacterial ribosome, for which Venkatraman Ramakrishnan, Thomas Steitz and Ada Yonath won the 2009 Nobel Prize.

Crystallography not only yields data on protein structure, but can visualize interactions between cofactors, ligands, and drugs if those compounds can be co-crystallized with the protein. One of the major limitations of crystallography is that crystallization conditions cannot be determined beforehand, and standard protocol is to optimize various solution variables in a combinatorial approach[139]. The crystallization is generally done in solution and must be done gradually, so as

to form diffraction-ready crystals and not amorphous aggregates. The precipitation conditions are more of a guessing game than anything, and random screening conditions seem to be more efficient than searches based on specific models[140]. Obtaining near-perfect crystals is of utmost importance especially in complex structures, as defects in the crystal structure lower the resolution at which the structure can be solved. As chemical bonds are no more than a few angstroms long, resolution below three angstroms is ideal[141]. Recently, efforts have been made to automate this labor-intensive process, with the goals of increasing both throughput and crystal quality[139].

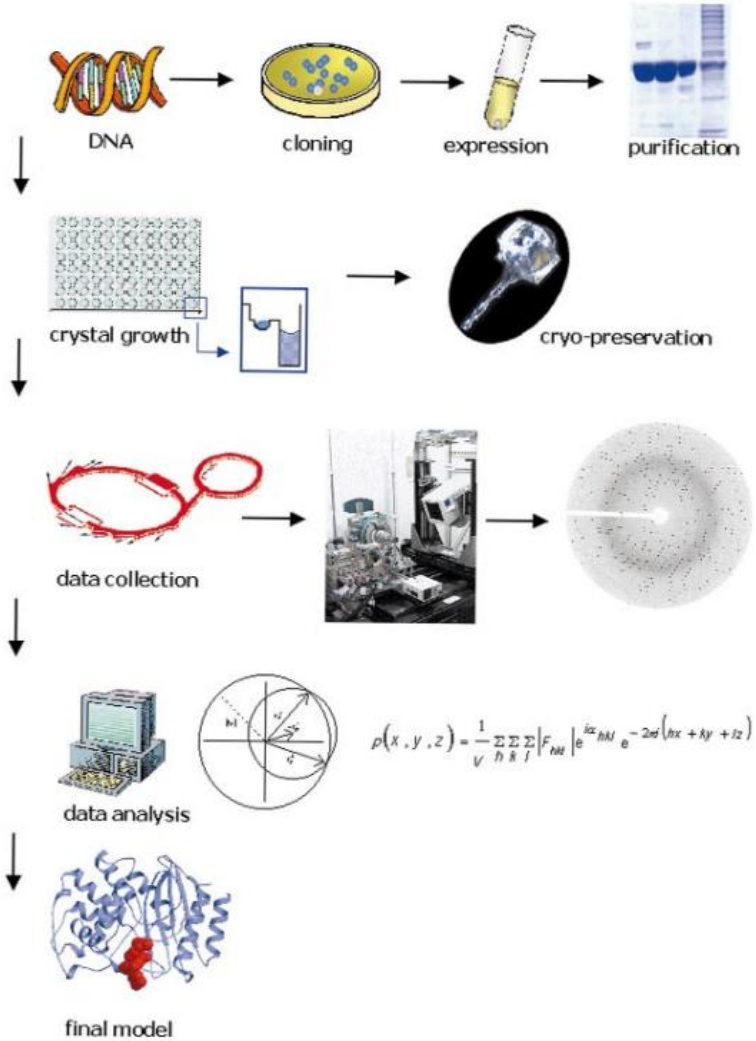


Figure 27. Creation of a computer-generated protein from a protein crystal. Adapted with permission from [140]

CHAPTER 3. TAMOXIFEN

Tamoxifen, specifically the Z-isomer (Fig 28), is a triphenylethylene compound and a non-steroidal antiestrogen which is used clinically for the treatment of estrogen receptor (ER) positive breast cancer, as well as for adjuvant therapies. Indeed, tamoxifen is the most widely used anticancer drug on the market today[142]. Although this is its primary use, tamoxifen was not developed with cancer treatment in mind, and indeed displays several other interesting properties, including antimicrobial effects. This chapter will cover a history of tamoxifen, an overview of both its primary and alternative functions, focusing on the antimicrobial properties, and contains the research I undertook to expand its antibacterial properties and understand its mechanism of function.

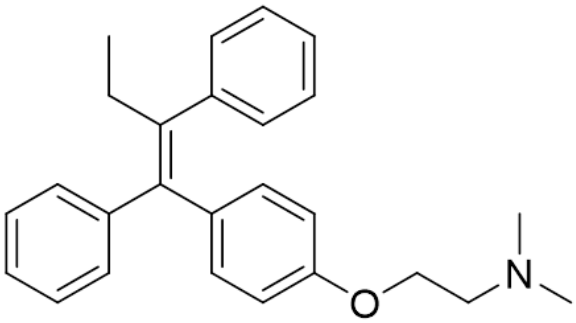


Figure 28. (Z)-tamoxifen

3.1 Tamoxifen: discovery, development and usage

3.1.1 History of tamoxifen

Tamoxifen was discovered and developed by Imperial Chemical Industries (ICI) in 1962 and was initially pursued as an antifertility drug, due to its effectivity as a post-coital contraceptive in mice[143]. Unfortunately, the compound showed the opposite effect in human trials, actually inducing ovulation. As research in the 1960's emerged linking estrogen to breast cancer, it was

suggested that tamoxifen, as an antiestrogen, might see some success as an anticancer agent. Arthur L. Walpole, the head of the fertility control program at ICI made efforts to push tamoxifen into medical use, supporting clinical tests in a Manchester institute. In 1973, tamoxifen was approved for use as a breast cancer treatment in the UK under the name Nolvadex[144]. Although it saw some success as an anticancer treatment, tamoxifen's true triumph was as an adjuvant therapy and a chemo-preventative[145]. Initial studies were relatively short term, only a few years, but extended or indefinite adjuvant therapy using tamoxifen showed the drug to be tumorstatic and prolonged dosing prevented metastasis[146]. There were worries about potential side-effects from long term treatment, such as bone loss and risk of heart disease, but tamoxifen in fact shows bone preservation and cholesterol decreases in postmenopausal women[142]. Tamoxifen opened the field of selective estrogen receptor modulators (SERMs), and led the way for the development of other SERMs such as raloxifene[147]. Tamoxifen, in addition to its anticancer properties, shows promise in a number of other areas, only a few of which will be mentioned, namely the antimicrobial variety. Tamoxifen, incidentally, is a protein kinase C inhibitor, which is implicated in several off-target biological effects of the drug[148].

3.1.2 Tamoxifen as an antifungal

Tamoxifen shows antifungal activity, with good MIC against pathogenic yeasts such as *Candidas* species as well as *Cryptococcus neoformans*. It was also shown to reduce kidney fungal burden in a murine animal model[149]. Tamoxifen functions by interfering with calmodulin, leading to increased calcium within the cells and affecting polarized growth. In *Cryptococcus,* this interference prevents the activation of a serine-threonine phosphatase called calcineurin, which is a virulence factor[150]. This is a promising treatment as compounds which

directly target calcineurin also act as immunosuppresants in humans, targeting calcineurin in T-cells. Tamoxifen and other triphenylethylenes are being pursued as potential treatments which attack pathogenic yeasts with few side effects.

3.1.3 Tamoxifen as an antileishmanial agent

Tamoxifen has antiparasitic properties, showing efficacy at low doses against *Leishman braziliensis* and *Leishman chagasi[151]*. The studies were done in rodent models and tamoxifen treatment was as effective as the control drug, reducing parasite load by over 95%. Additionally, the subjects suffered no ill effects and had a 100% survival rate whilst every negative control died by 18 weeks. Current leishmanial treatments are primarily antimony based, toxic, require daily injections, and are losing effectivity due to the rising occurrence of resistance. Miltefosine is a possible alternative, showing good effectivity and boasts oral availability[152]. Tamoxifen shows promise as a safer, more effective treatment. The drug was found to basify the vacuoles of the parasite, and the mechanism was suggested to be inhibition of proton-dependent ATPase, although more research is required to verify the verisimilitude of this mechanism[153].

3.1.4 Tamoxifen as an antibiotic

Finally, tamoxifen has antibacterial properties. The compound shows favorable MICs towards gram-positive bacteria such as *Staphylococcus aureus*, however, the mechanism is poorly understood at best. It was demonstrated that tamoxifen disrupts the membranes of a model microbe, *Bacillus stearothermophilus* (Fig 29), causing ultrastructural alterations leading to ion leakage[126]. This study reached the conclusion that tamoxifen was a membrane-active drug and that membrane damage was the primary mechanism of action for tamoxifen. While the data certainly suggests membrane effects, the exploration of the mechanism was cursory at best,

content to confirm membrane activity and leave it at that. In reality, the mechanism of the antibacterial activity of tamoxifen may be more complex. While it is certainly feasible for a non-specific interaction to occur, it is also possible that a more targeted interaction is occurring. Very little research has been carried out with the goal to examine and enhance the antimicrobial properties of tamoxifen.

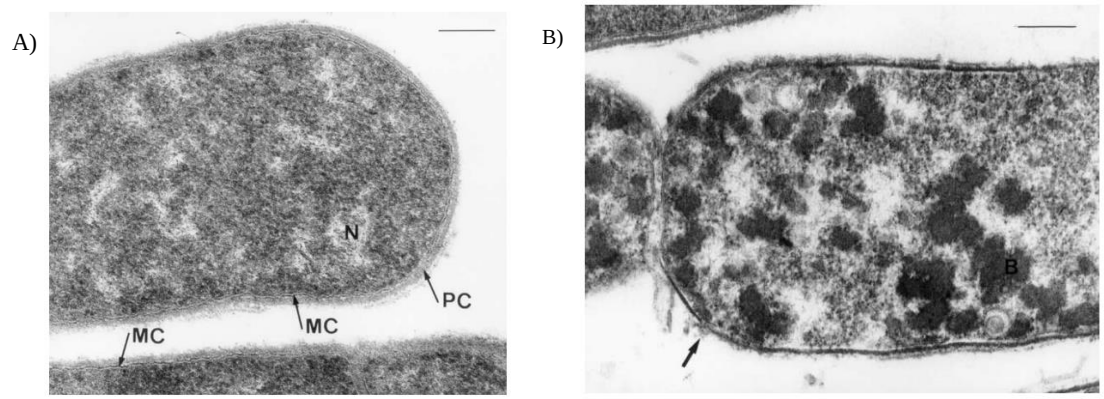
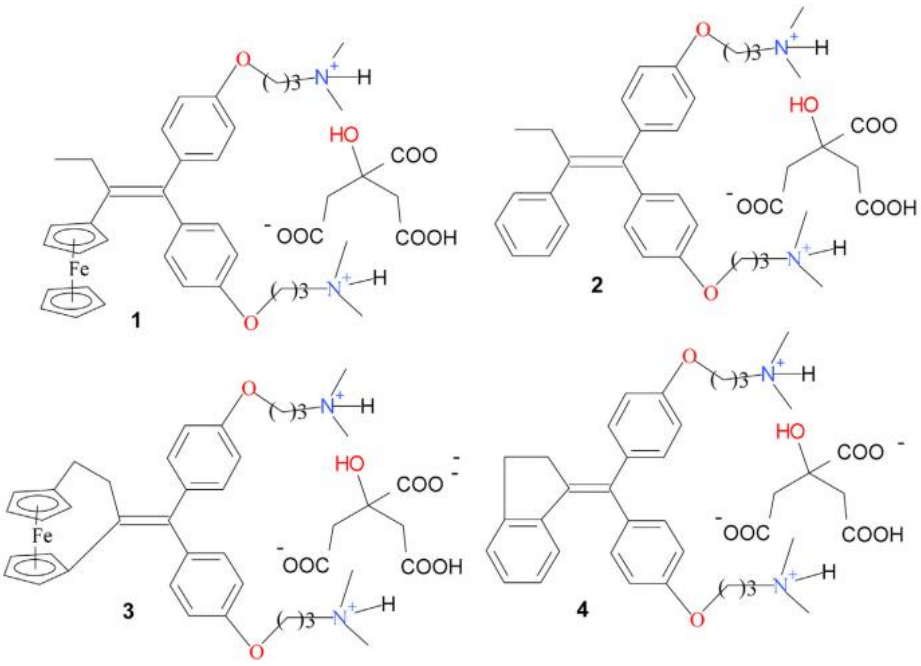


Figure 29. A) untreated B. stearothermophilus. MC indicates intact membrane profile and PC indicates intact cell wall B) B. stearothermophilus treated with 10 µM tamoxifen for 140 min. arrow indicates cell wall leakage. Adapted with permission from [127]

One of the few studies which did examine tamoxifen by proxy assessed the antibacterial properties of ferrocenyl analogs of tamoxifen derivatives, bearing a second alkyl amino chain[154]. It was concluded that there was no general correlation between the antitumoral and antibacterial properties of a given tamoxifen derivative. The addition of the second alkyl amino chain does not appear to enhance antibacterial activity much beyond standard tamoxifen, however the replacement of one of the phenyls with a ferrocene group increased the MIC by a factor of 4 for *Staphylococcus aureus* (Fig 30). This study likewise looked at TEM images of cells before and after treatment, noting membrane damage associated with treatment with the tamoxifen derivatives. This study also noted significant ion efflux upon treatment, up to 95% of intracellular potassium in *Listeria sp.*



Strains	Minimum inhibitory concentration MIC (μM)			
	Compound **1**	Compound **2**	Compound **3**	Compound **4**
Listeria ivanovii HPB28	12.5	25	12.5	50–25
Listeria monocytogenes LSD 532	8	31	16	31
Listeria monocytogenes LMA 1045	8	62	8–16	62
Enterococcus faecalis ATCC 27275	8	80	20	40
Staphylococcus aureus ATCC 6538	8	31	8	31
Escherichia coli MC4100	100–50	50	100	100
Escherichia coli ATCC 11229	50	100–50	125	100
Pseudomonas aeruginosa ATCC 15442	>5000	>5000	>5000	5000–2500
Salmonella enterica ATCC 14028	625	1250	625	2500

Figure 30. Citrate salts of tamoxifen analogues and their MICs against various bacterial strains. Adapted with permission from [155]

A more recent study of tamoxifen as an antibacterial found that it showed effectiveness against *Mycobacterium tuberculosis*, showing equivalent effectivity against multi-drug resistant and extreme resistant strains and the standard strain[155]. The concentrations at which tamoxifen

was effective was well below the 50% cytotoxicity level for murine macrophages, indicating it may be an effective treatment with minimal off-target toxicity. This study did not explore the mechanism of tamoxifen's antibacterial activity, but the researchers intend to proceed with testing of tamoxifen derivatives against tuberculosis. As can be seen from the ubiquity of tamoxifen, the compounds is a prime target for an SAR on the antibiotic effects of tamoxifen. The compound has well defined areas – the triphenyl portion, stereospecificity, and a terminal amine chain – making it straightforward to approach from a design standpoint. The literature is lacking in strong evidence on the effects of altering the tamoxifen structure with regards to its antibacterial activity, and such a study will help elucidate the mechanism, to determine whether it is mere membrane effect or in actuality a targeted effect. Therefore, a study examining minor alterations to the structure of tamoxifen was undertaken and the results are presented in the following chapter.

CHAPTER 4. STRUCTURE-ACTIVITY RELATIONSHIP OF TAMOXIFEN

4.1 Purpose of study

In order to clarify the mechanism of tamoxifen as well as to enhance its antimicrobial properties, an SAR study of tamoxifen was undertaken. This SAR study was complemented and guided by MIC testing of compounds to evaluate their antibacterial activity against *Staphylococcus aureus* (SA) as well as a methicillin-resistant strain (MRSA). This study was approached through the hypothesis that Tamoxifen, a non-steroidal antiestrogen, displays antibacterial properties through an unclear mechanism. It was believed that the antiestrogenic properties of tamoxifen could be decoupled from the antibacterial properties of tamoxifen, converting an anticancer agent with Estrogen receptor (ER) specificity into a general antibiotic and that the process of structural modifications taken to achieve it would help elucidate the unclear mechanism by which tamoxifen displays antibacterial properties. Although the SAR did not get to the point of decoupling ER-specificity, a good number of derivatives were designed, synthesized, and tested, informing of possible mechanistic leanings.

4.2 Significance

This research is of high significance because the looming threat of antibacterial resistance makes the pursuit of novel compounds imperative. Constant reports of new multi-resistant bacteria and a drying stream of new antibiotics along with the immense economic burden of infection by resistant microbes emphasize the urgent need for research into new antibacterial compounds. Tamoxifen displays antibacterial activity through an unclear mechanism and may possibly represent a new

class/subclass of antibiotics, making the evaluation, optimization, and mechanistic elucidation of this compound tremendously worthwhile.

4.3 Synthesis of tamoxifen derivatives

NMR and mass spectrometry data for each compound is located in appendix II. Compound structures and MIC data is compiled in Table 1, in the results section.

4.3.1 Preparation of N-desmethyl tamoxifen

Tamoxifen (Cayman Chemical) and 1-chloroethylchloroformate (Sigma-Aldrich, St. Louis, MO) were refluxed in dichloromethane (DCM) at 80^{o} C overnight. The reaction mixture was concentrated *in vacuo* and resuspended in hexanes to precipitate unreacted tamoxifen, which was then filtered off. The chloroformate product was concentrated, then refluxed in methanol (MeOH) for 3 hr at 90^{o}. N-desmethyl tamoxifen was recovered in 91% yield.

4.3.2 Preparation of extended-alkyl derivatives

N-desmethyl tamoxifen and a terminal bromoalkane were refluxed at 80^{o} C overnight in DCM in the presence of Hunig's base (N,N-diisopropylethylamine) in a pressure tube. The reaction was purified by preparative TLC. Bromopropane and bromohexane were utilized together with underivatized tamoxifen and desmethyl tamoxifen, created an alkyl chain series of C0 (desmethyl tamoxifen), C1 (tamoxifen), C3 (propyl derivative), and C6 (hexyl derivative). Yield of the propyl derivative was 76%, yield of the hexyl derivative was 81%.

4.3.3 N-acetyl tamoxifen

N-desmethyl tamoxifen was mixed with acetic anhydride in DCM for 24 hr at room temperature. TLC showed complete consumption of the starting material and drying under high vacuum was

sufficient to remove excess acetic anhydride and acetic acid. NMR of this compound showed a unique isomerization, thought to be across the N-C-O bonds, which resulted in near symmetrical peak splitting. Upon heating, the peaks reintegrated to match what was expected, as heating drove the racemic mixture towards a single isomer. This peak splitting was present in every subsequent derivative which contained the N-C-O bond, namely all the amino acid derivatives.

4.3.4 Glycine derivative

N-Boc protected glycine was mixed with N,N-dicyclohexylcarbodiimide (DCC) in DCM for 30 min at 0 ^{o}C. This yielded an amino anhydride and precipitated reacted DCC as dicyclohexylurea (DHU). This mixture was filtered and N-desmethyl tamoxifen was added. The reaction mixture was refluxed overnight in a pressure tube. The Boc-product was purified using preparative TLC and deprotected in 10% TFA/DCM for 3 hr at room temperature then dryed *in vacuo*, affording the final product 49% yield.

4.3.5 Alanine derivative

N-Boc protected alanine was mixed with N,N-dicyclohexylcarbodiimide (DCC) in DCM for 30 min at 0 ^{o}C. This yielded an amino anhydride and precipitated reacted DCC as dicyclohexylurea (DHU). This mixture was filtered and N-desmethyl tamoxifen was added. The reaction mixture was refluxed overnight in a pressure tube. The Boc-product was purified using preparative TLC and deprotected in 10% TFA/DCM for 3 hr at room temperature then dryed *in vacuo*, affording the final product 35% yield.

4.3.6 Phenylalanine derivative

N-Boc protected phenylalanine was mixed with N,N-dicyclohexylcarbodiimide (DCC) in DCM for 30 min at 0 ^{o}C. This yielded an amino anhydride and precipitated reacted DCC as

dicyclohexylurea (DHU). This mixture was filtered and N-desmethyl tamoxifen was added. The reaction mixture was refluxed overnight in a pressure tube. The Boc-product was purified using preparative TLC and deprotected in 10% TFA/DCM for 3 hr at room temperature then dryed *in vacuo*, affording the final product 42% yield.

4.3.7 Lysine derivative

Initial coupling was achieved as described above using Fmoc-Lys(Boc)-OH. Boc deprotection was achieved as described above. After concentration *in vacuo*, the Fmoc-protected product was reacted with neat piperidine for 2 hr at room temperature, the reaction was monitored via TLC. Excess piperidine was removed *in vacuo* and the reaction mixture was purified via preparative TLC. The purified compound was obtained in 11% yield.

4.3.8 Guanidine derivative

di-Boc-guanidyl triflate was mixed with N-desmethyl tamoxifen in the presence of TEA in DCM. The reaction mixture was refluxed overnight. The Boc-product was purified via preparative TLC and deprotected in 10% TFA/DCM for 3 hr. The product was recovered at a yield of 19%.

4.3.9 Didesmethyl tamoxifen and 4-hydroxy tamoxifen

N,N-didesmethyl tamoxifen was purchased from Toronto Research Chemicals. 4-hydroxy tamoxifen, the major metabolite in humans, was purchased from EMMX. Solutions were prepared from the compounds as purchased.

4.4 Compound assessment

Compounds were assessed using MIC assays as described in Chapter 2. MIC_{50} assays were performed by λ600 absorbance in 96-well plates according to CLSI protocol. *S. aureus* ATCC

29213 and Methicillin-resistant *S. aureus* ATCC 33591 were cultured in tryptic soy broth (TSB) (17g tryptone, 3g soytone, 2.5g dextrose, 5.0g NaCl, 2.5g K_2HPO_4 in 1L DI water) at 37 ^oC. Each sample was tested in triplicate with both drug and vehicle controls. MIC values were determined using Graphpad Prism 6 software.

4.5 Results

MIC traces are located in appendix C.

Table 1. Structures and MICs

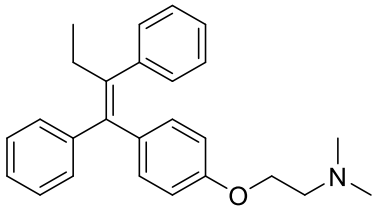
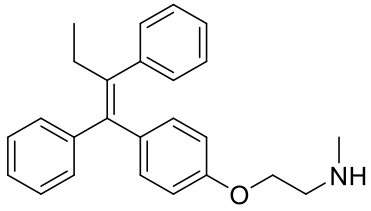
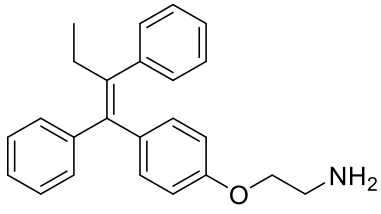
Compound	Structure	MIC_{50} SA (µg/mL)	MIC_{50} MRSA (µg/mL)
Tamoxifen		4.29±0.07	7.92±0.09
Desmethyltamoxifen		3.45±0.45	5.37±0.19
Didesmethyltamoxifen		2.60±0.03	4.64±0.6

Table 1 continued

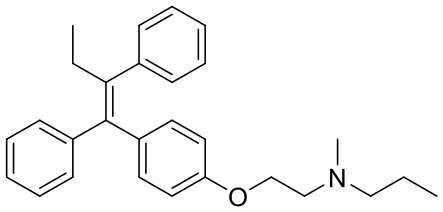
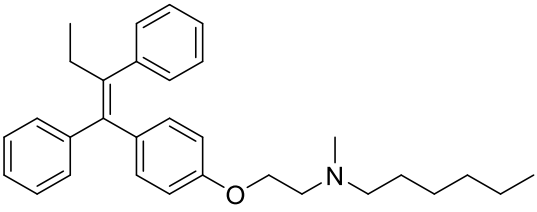
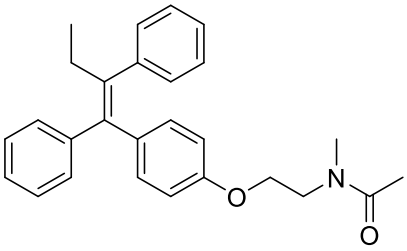
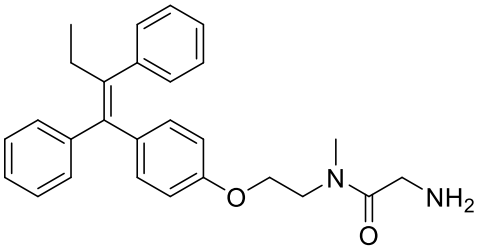
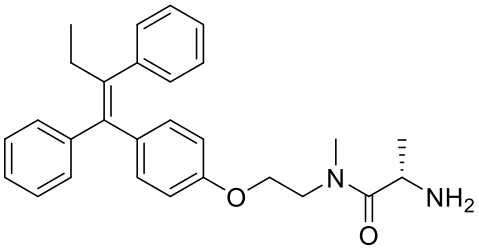
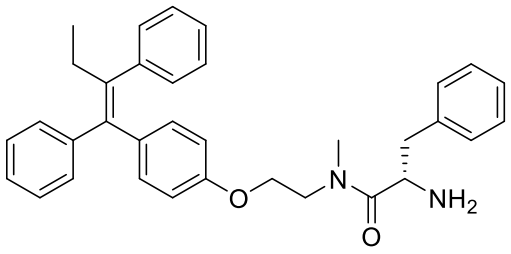
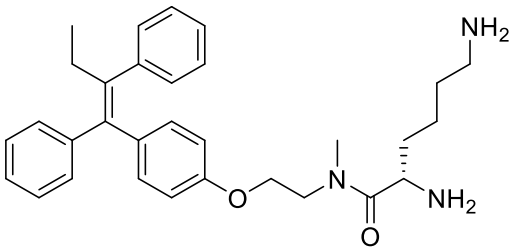
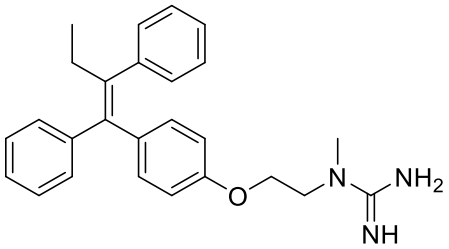
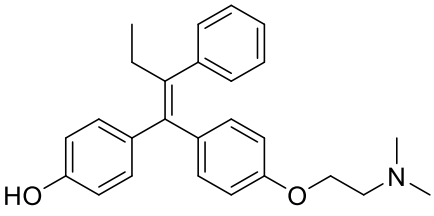
NL-I-44		11.5±2.7	NI
NL-I-43		NI	NI
NL-I-45		NI	NI
NL-I-72		8.78±0.09	14.51±1.4
NL-I-70		8.14±0.19	9.55±0.05

Table 1 continued

NL-I-71		6.82±0.59	5.91±0.13
NL-I-97		14.62±0.73	3.38±0.14
NL-I-69		2.06±0.2	2.5±0.06
4-hydroxytamoxifen		NI	NI

4.6 Discussion

Although the SAR was attenuated before the triphenyl portion could be altered, there are important gleanings within the results presented. The extension of the alkyl chain highlights the importance of the positive charge on the terminal amine and begins to expose the detriment of added hydrophobicity. A clear correlation between alkyl chain length and activity is seen, where

didesmethyl tamoxifen is the most active compound, followed by desmethyl tamoxifen and tamoxifen. Extending the alkyl chain even to three units hampers activity in SA and abolishes activity in MRSA. Extending the chain to six units completely obliterates the activity even in SA. This data suggests that there is a fine balance between the degree of hydrophobicity of the alkyl groups attached to the tamoxifen amino group and its antibacterial activity. Additionally, the positive charge which the amine adopts *in vivo* has a tremendous effect on activity. This is further corroborated by the acetyl and glycine derivatives. The acetyl group effectively mutes the positive charge and is completely inactive. The glycine derivative, which essentially restores the positive charge and terminal amine, has restored activity equivalent to underivatized tamoxifen. As bulk is added to the alpha-carbon, it seems to have a minimal effect on activity. In almost every case, the compounds are less effective in MRSA. The two exceptions to this are the phenylalanine derivative, which is not significantly different in the SA as in the MRSA, and the lysine derivative, which alone shows almost a 5-fold decrease in MIC in the MRSA. Interestingly, the guanidine derivative is the most effective. Guanidine may have increased activity due to its relative basicity compared to the standard tamoxifen tertiary amine. Although no precise mechanism can be suggested from the findings, some insights are clearly suggested. The hydrophilicity of the amine end plays a key role in the mechanism, as attenuation and muting of the charge abolishes activity. A relatively undecorated derivative is most desirable, as in every case increasing the bulk on the amine end decreased activity, even in the case of lysine which offered an extra positive charge. While these findings do not conclusively point to a targeted interaction, they by no means rule it out and can even be considered possible evidence of a targeted interaction through the positive charge of the amine. Given the previous research which suggests membrane activity however, it is feasible that tamoxifen functions in a manner similar to polymyxins, which have hydrophobic tail

and cationic peptide section, acting as detergents and leading to ion efflux. It is also reasonable to suggest the mechanism may be closer to that of lipopeptides, with the hydrophobic portion inserting into the membrane, resulting in porosity of the membrane, resulting in ion efflux. However, the inactivity of 4-hyrdoxy tamoxifen may indicate the interruption of a specific, targeted reaction, which is in line with such a modest alteration causing such dramatic effects. In either case, minimal alterations to the tamoxifen amine are desirable, as evidenced particularly by the lysine derivative losing activity. There is much work to be done, first and foremost to attempt decoupling tamoxifen's estrogen-receptor affinity from its antibacterial activity. With this initial groundwork in place, it is my hope that this project can be continued as new antibiotics are desperately needed and tamoxifen may be a doorway to novel compounds, as it has been in so many other fields.

APPENDIX A. COLLABORATION WITH DR. DEV ARYA

In collaboration with Dr. Dev Arya of Clemson University, aminoglycosides were linked to anthraquinone to limit natural defenses in the form of aminoglycoside modifying enzymes (AMEs) from altering the ribosome binding aminoglycoside portion. All compounds were synthesized by the Arya lab, and were put through a variety of tests, including a cell-free ribosomal inhibition assay.

A.1 Methods

The cell-free ribosomal inhibition assay is performed by treating E. coli cell extracts with compounds for testing followed by incubation with cDNA, leading to translation of a reporter protein, in this case luciferase. E. Coli S30 extract kits were obtained from Promega. S30 extract, S30 premix, amino acids, and pBESTluc circular DNA were thawed on ice. A Master Mix (MM) was created by combining 180 µL S30 premix, 135 µL S30 extract, 95 µL nanopure water, and 40 µL amino acid mix for a total volume of 450 µL. One tube of MM was sufficient for each compound run at 10 concentration points with 3 vehicle controls. Compounds were serially diluted in DMSO so that the final concentrations ranged from 1.25 µM to 2.4nM. The pBESTluc cDNA provided in the kit was diluted from 10 µL to 54.4 µL with sterile 1x TE buffer. 12.5 µL of MM were aliquoted to a 1.5 mL centrifuge tubes, followed by 0.5 µL compound. The tubes were then mixed and centrifuged briefly. The tubes were held at room temperature for 20 min, after which 0.4 µL pBESTluc solution was added. After gentle mixing and centrifugation, the tubes were incubated at 37^{o} C for 60 min. The tubes were put on ice for a 5-minute inactivation period. After gentle mixing by pipette, 5 µL were aliquoted to a white half-volume 96-well plate (Greiner). 35 µL 1mM luciferin solution (Promega) was added to each

well, and the plate was read for luminescence after a 30s-shaking period. Luminescence was normalized to DMSO controls. Data was processed with Graphpad Prism 6.

To verify that the compounds only showed activity in a prokaryotic ribosome, a similar experiment was carried out in a rabbit reticulocyte system. Rabbit Reticulocyte lysate kits were obtained from Promega. Cell lysate, RNasin, amino acids, and RNA were thawed on ice. Master Mix (MM) was created by combining 300 µL cell lysate, 5 µL RNasin, 137 µL nanopure water, and 8 µL amino acid mix for a total volume of 450 µL. One tube of MM was sufficient for each compound run at 10 concentration points with 3 vehicle controls. Compounds were serially diluted in DMSO so that the final concentrations ranged from 4.2 µM to 5nM. 12.5 µL of MM were aliquoted to a 1.5 mL centrifuge tubes, followed by 0.5 µL compound. The tubes were then mixed and centrifuged briefly. The tubes were held at room temperature for 20 min. after which 0.4 µL RNA was added. After brief mixing and centrifugation, the tubes were incubated at 30^{o} C for 90 min. The tubes were put on ice for a 5-minute inactivation period. After gentle mixing by pipette, 5 µL were aliquoted to a white half-volume 96-well plate (supplied by Greiner). 35 µL 1mM luciferin solution (Promega) was added to each well, and the plate was read for luminescence after a 30s shaking period. Luminescence was normalized to DMSO controls. Data was processed with Graphpad Prism 6.

Most compounds showed excellent activity against the prokaryotic ribosome, with IC_{50} values reaching low nanomolar values. The most active compounds were verified to have no activity in the eukaryotic ribosomes at the highest tested concentrations.

A.2 Results

Table A1. Cell-free IC_{50} Data

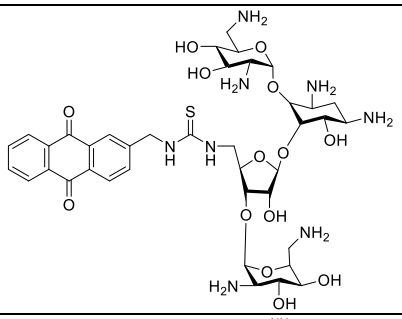
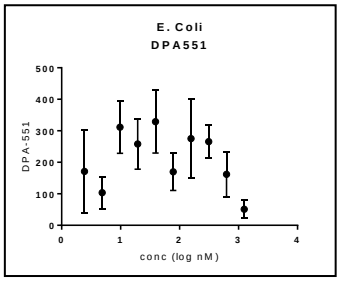
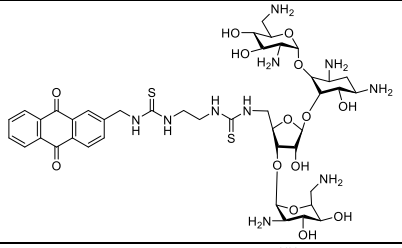
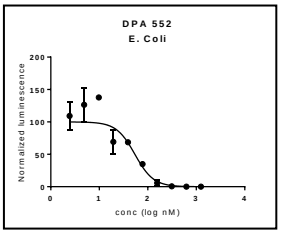
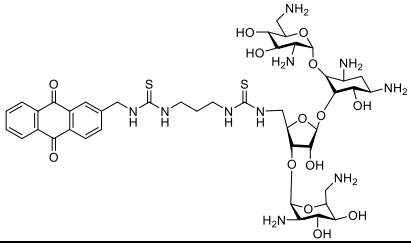
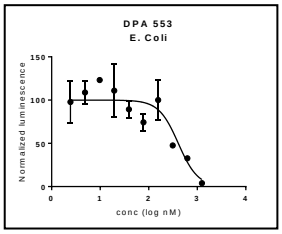
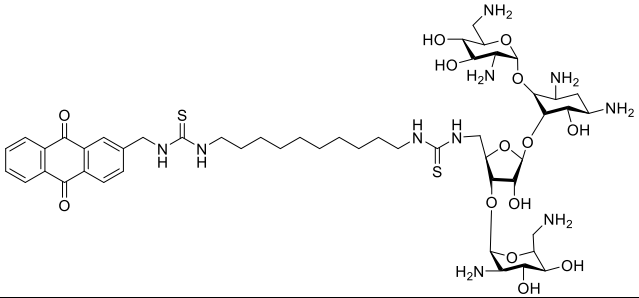
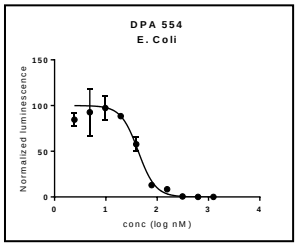
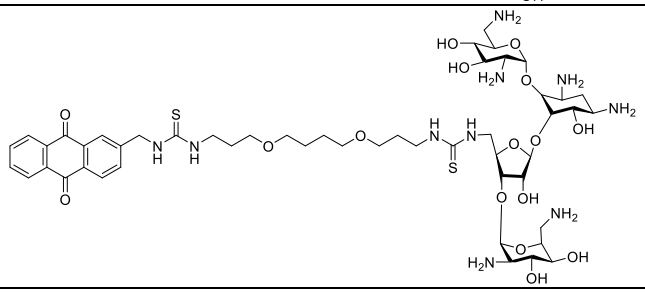
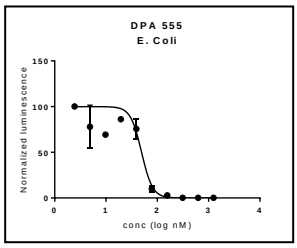
Compound	Structure	Cell Free Data	IC_{50} (nM)
DPA551			NI
DPA552			50±8.8
DPA553			303±28
DPA554			40.3±6.7
DPA555			35.4±3.5

Table A1 continued

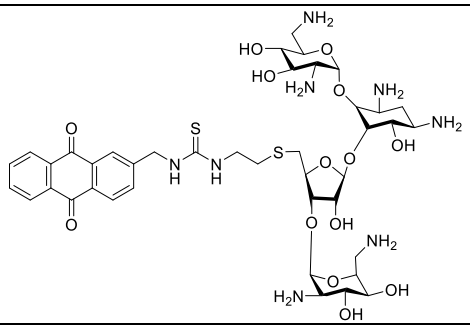
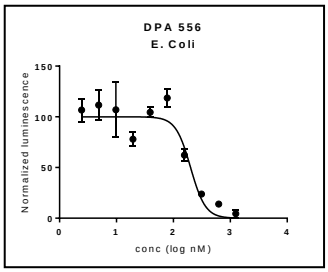
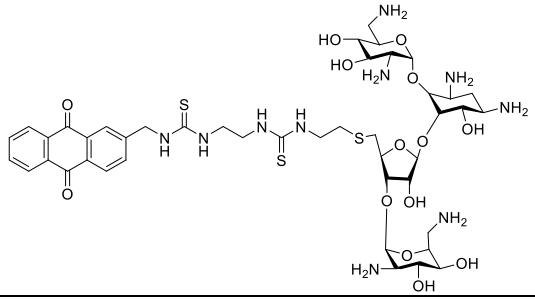
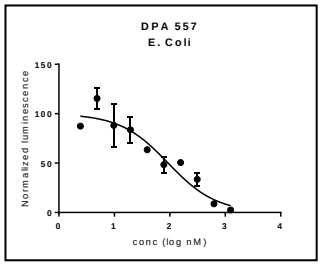
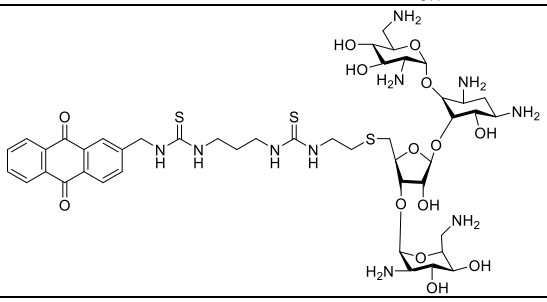
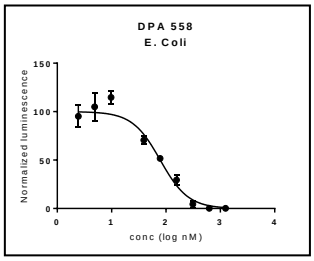
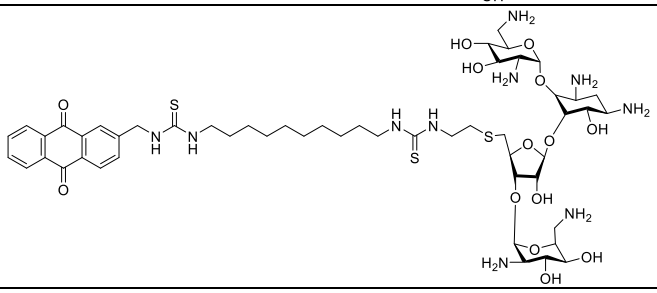
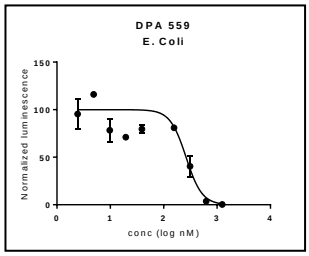
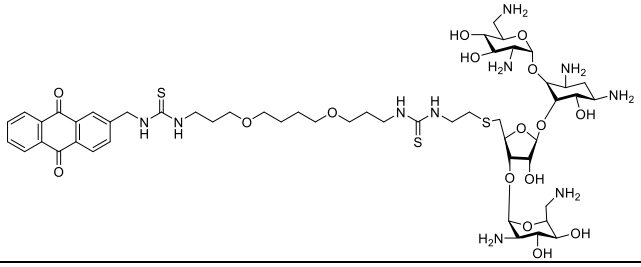
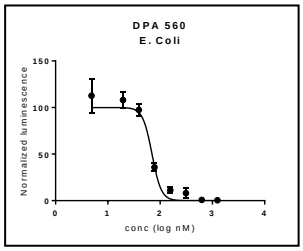
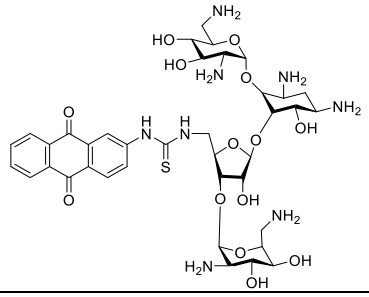
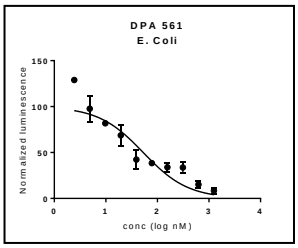
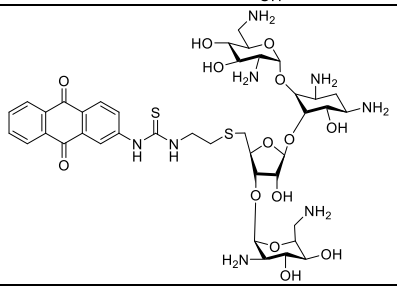
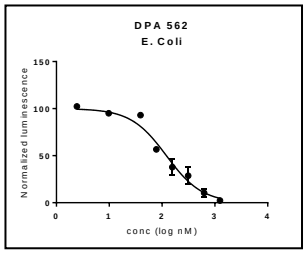
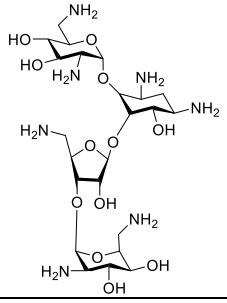
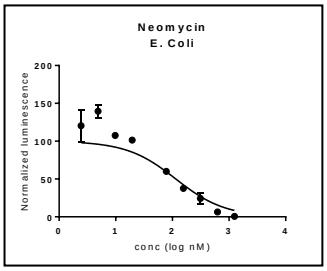
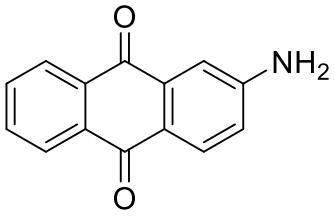
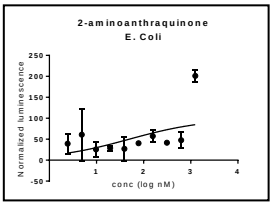
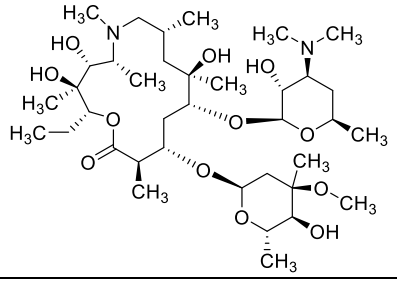
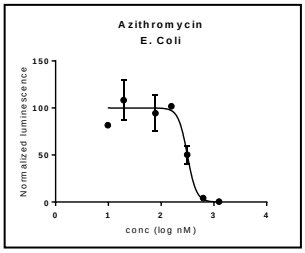
DPA556			178±18
DPA557			91±1.7
DPA558			101±16
DPA559			250±35
DPA560			71.4±2

Table A1 continued

DPA561			63.7±8.5
DPA562			164±8
Neomycin			139.7±10
2-amino anthraquinone			NI
Aziththromycin			337.1±37

APPENDIX B. NMR AND MASS SPECTROSCOPY DATA

B.1 NMR

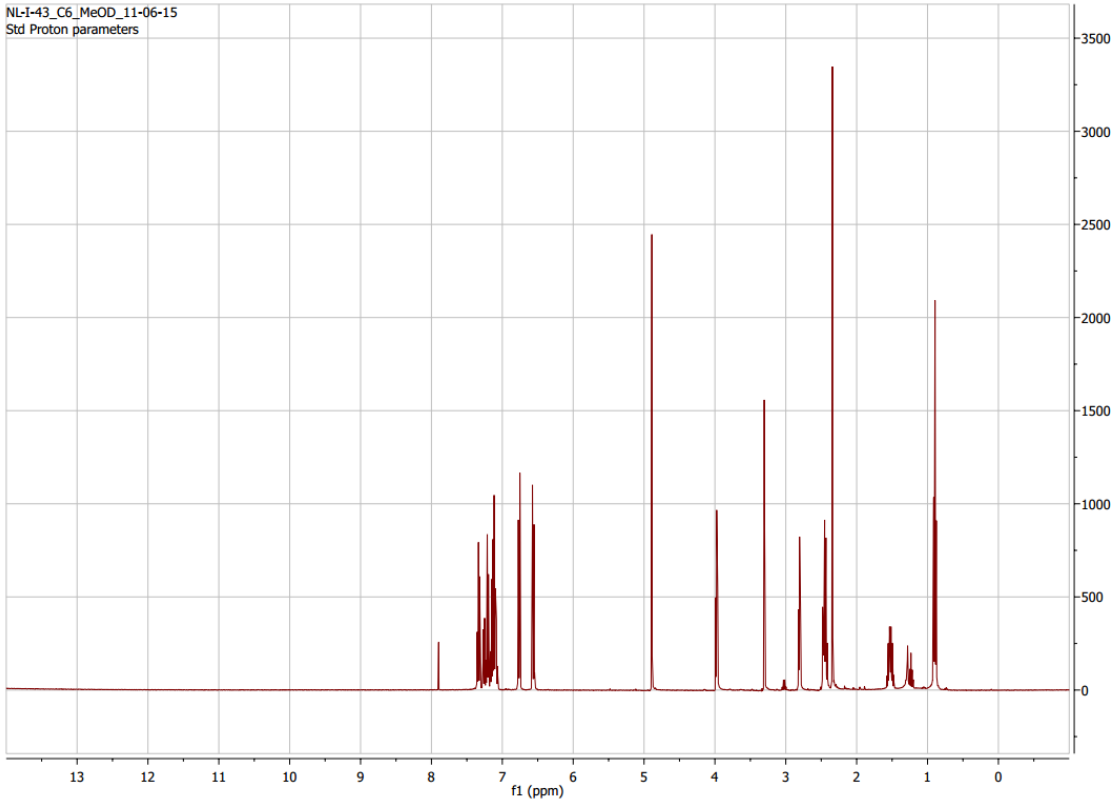


Figure B1. H^1 NMR of NL-I-43

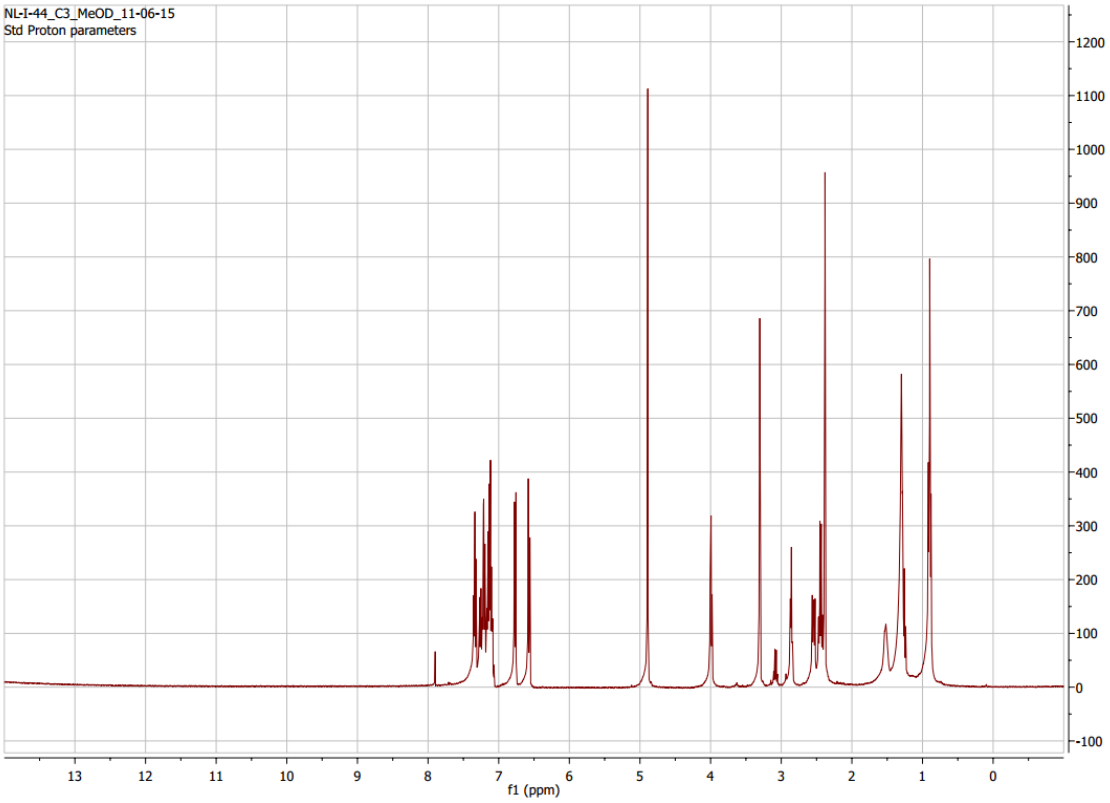


Figure B2 H^1 NMR of NL-I-44

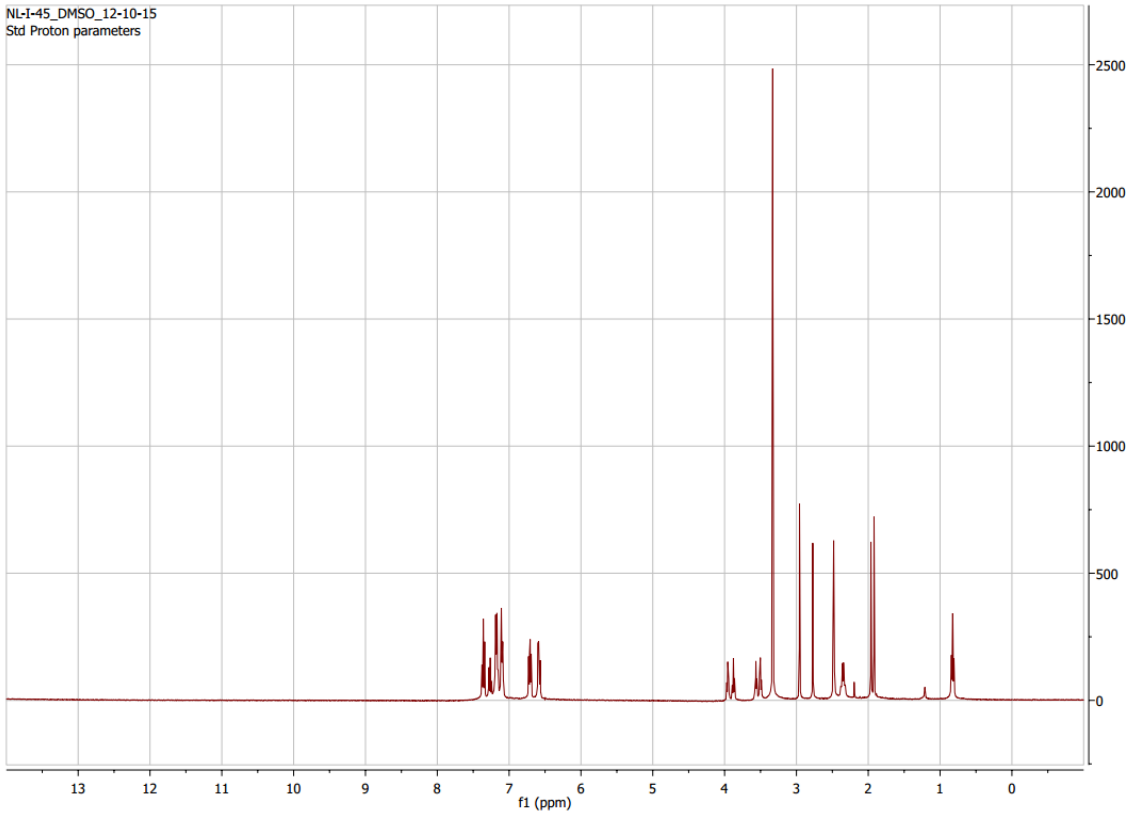


Figure B3 H^1 NMR of NL-I-45

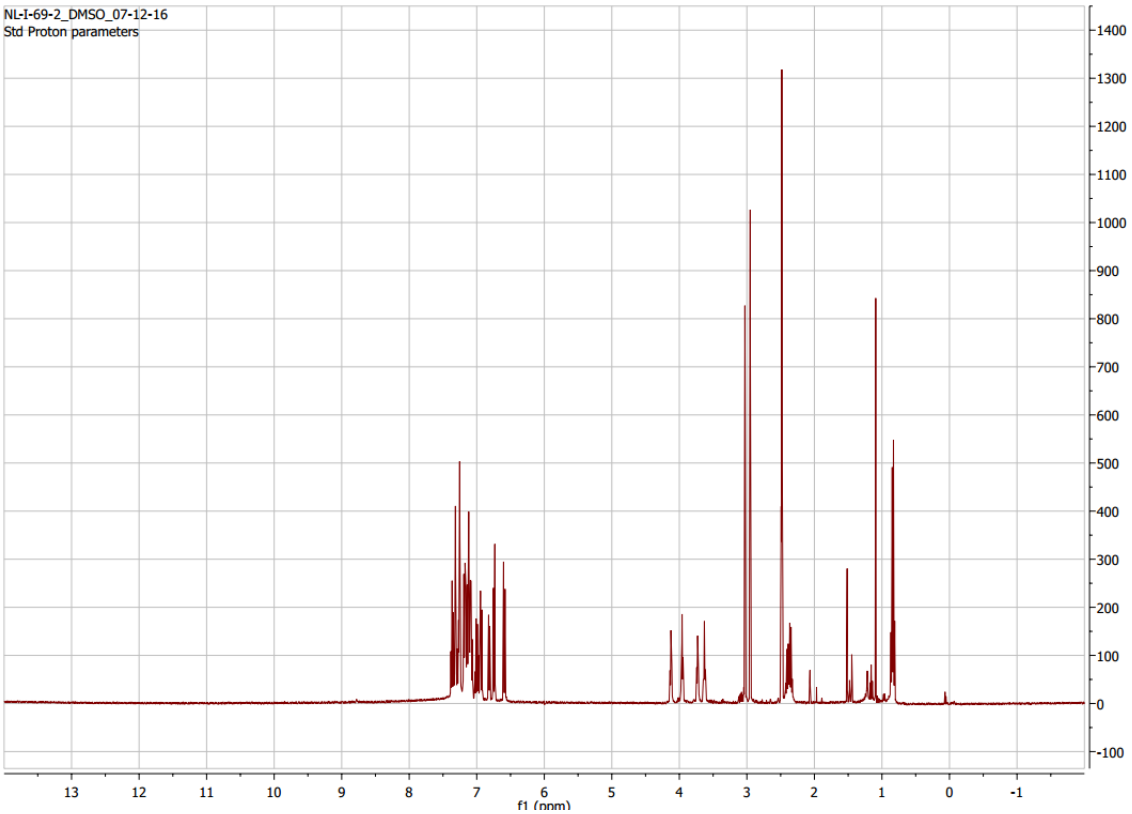


Figure B4 H^1 NMR of NL-I-69

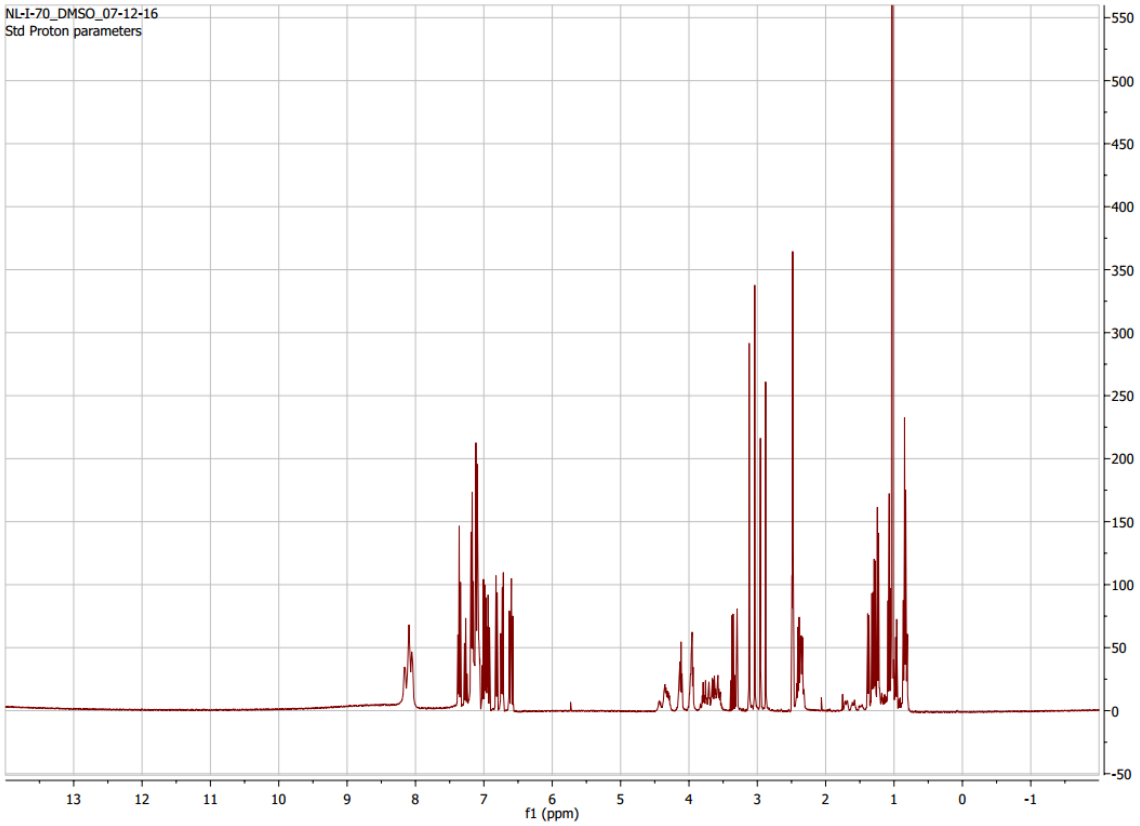


Figure B5 H^1 NMR of NL-I-70

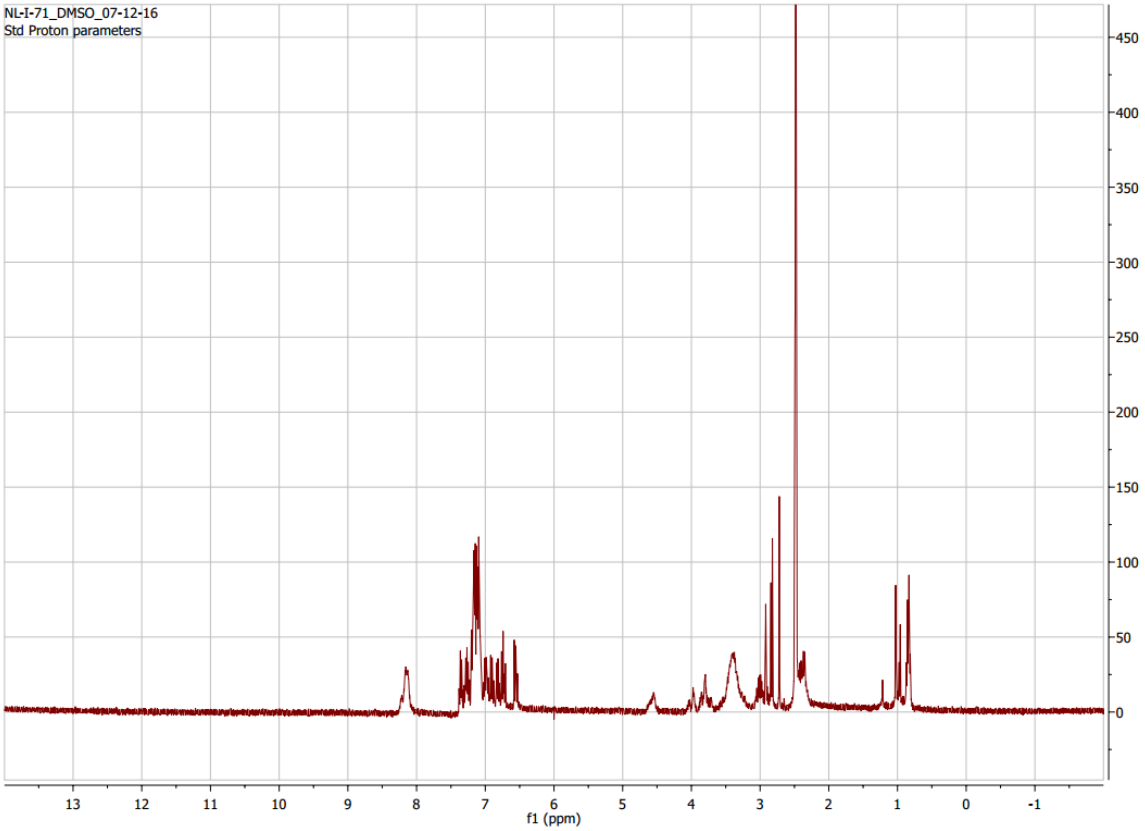


Figure B6 H^1 NMR of NL-I-71

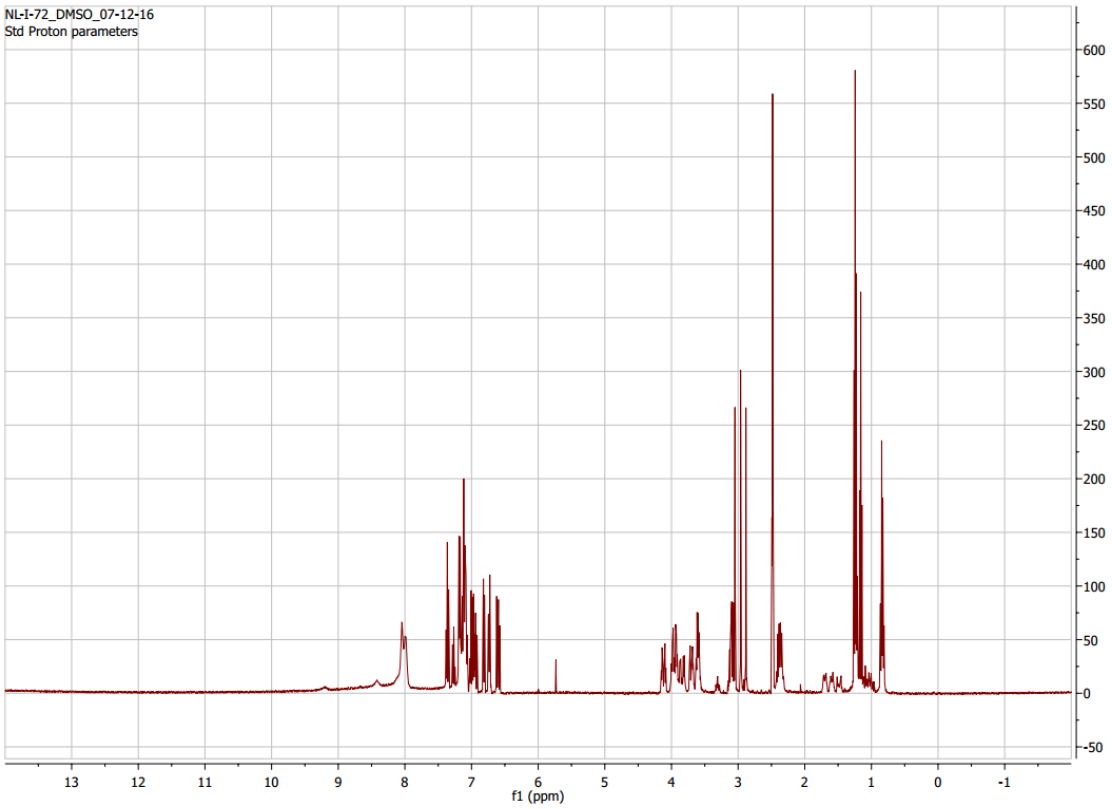


Figure B7 H^1 NMR of NL-I-72

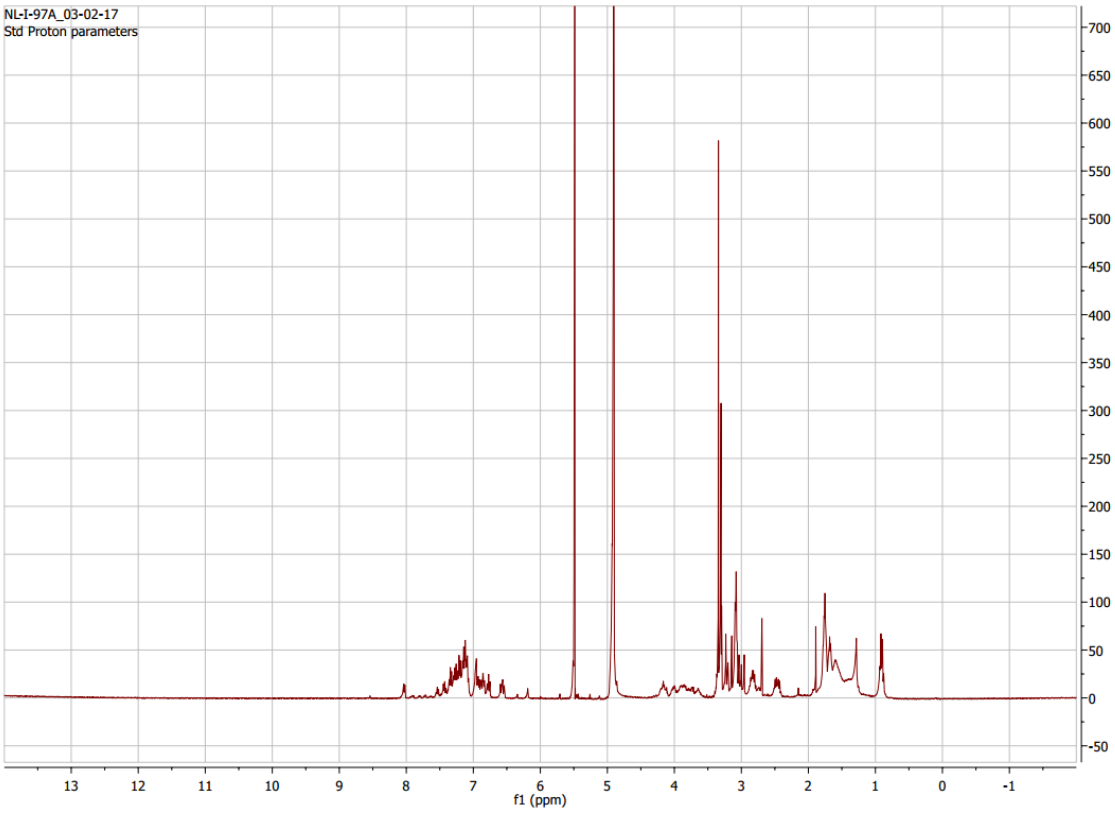


Figure B8 H^1 NMR of NL-I-97GT mass spec la

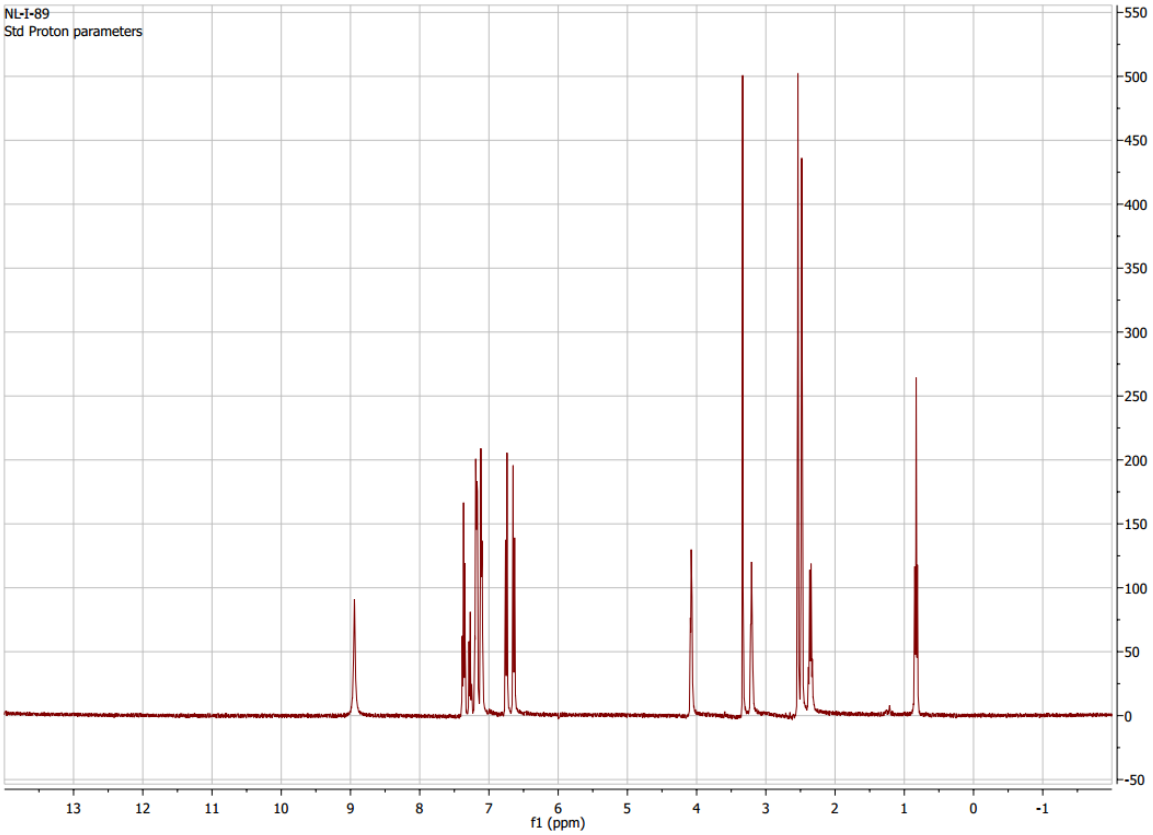


Figure B9 H^1 NMR of Desmethyl tamoxifen

B.2 Mass Spectrometry

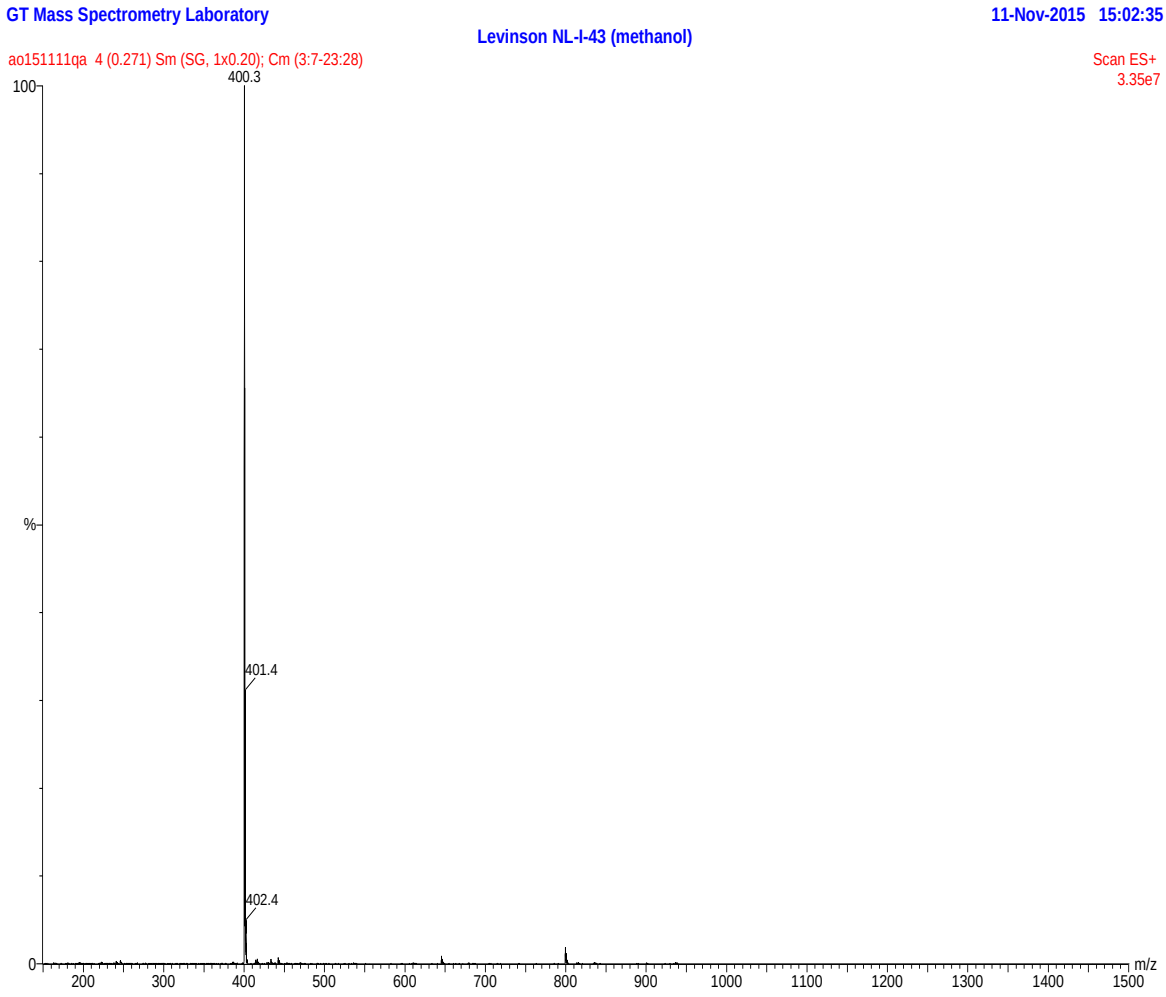


Figure B10 Mass spec of NL-I-43

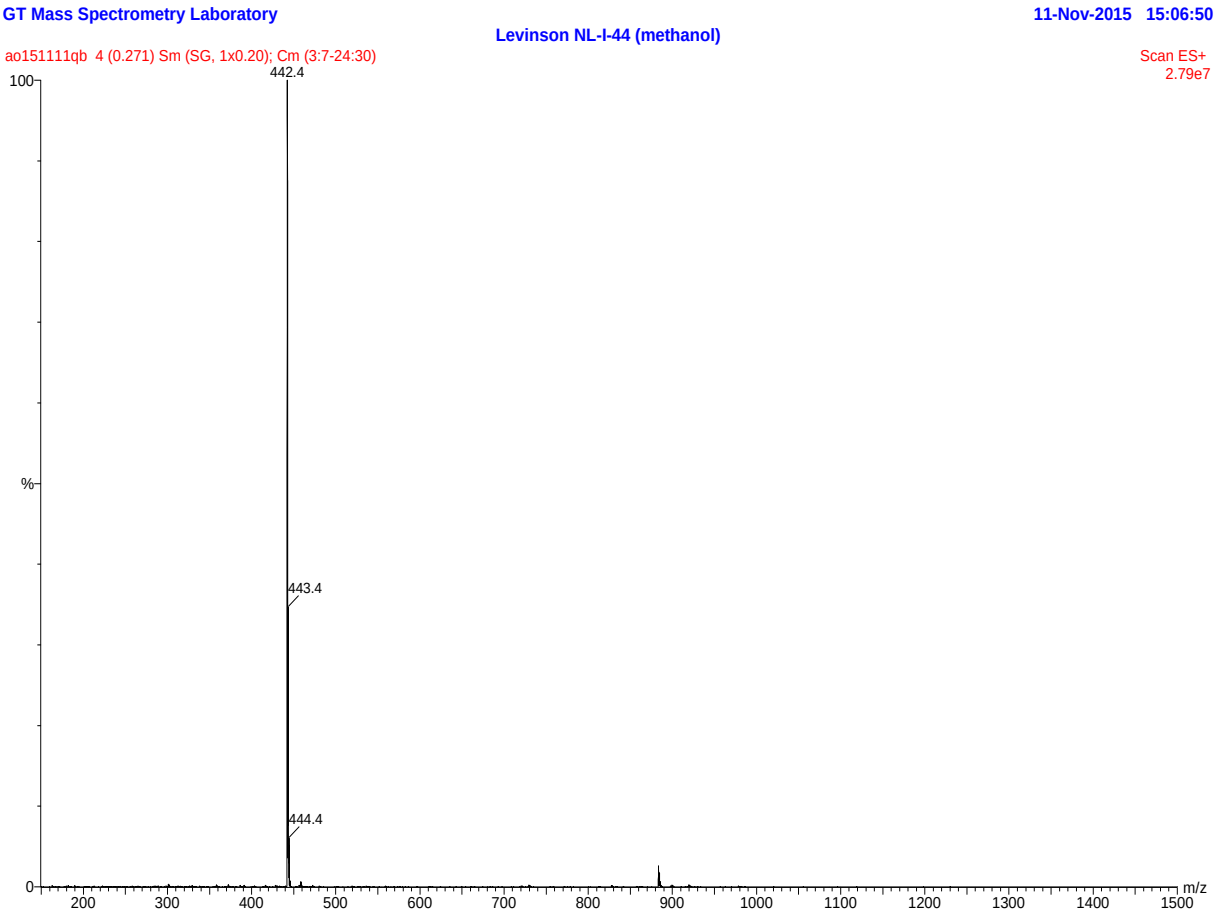


Figure B11 Mass Spec of NL-I-44

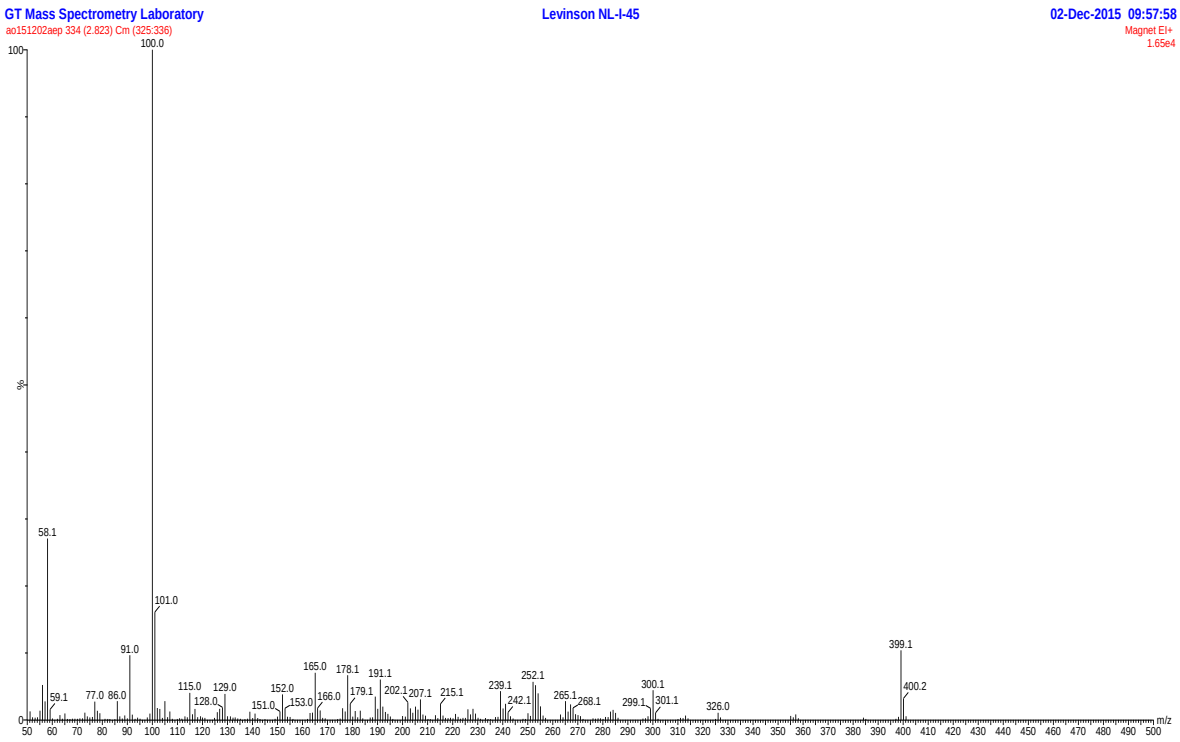


Figure B12 Mass spec of NL-I-45

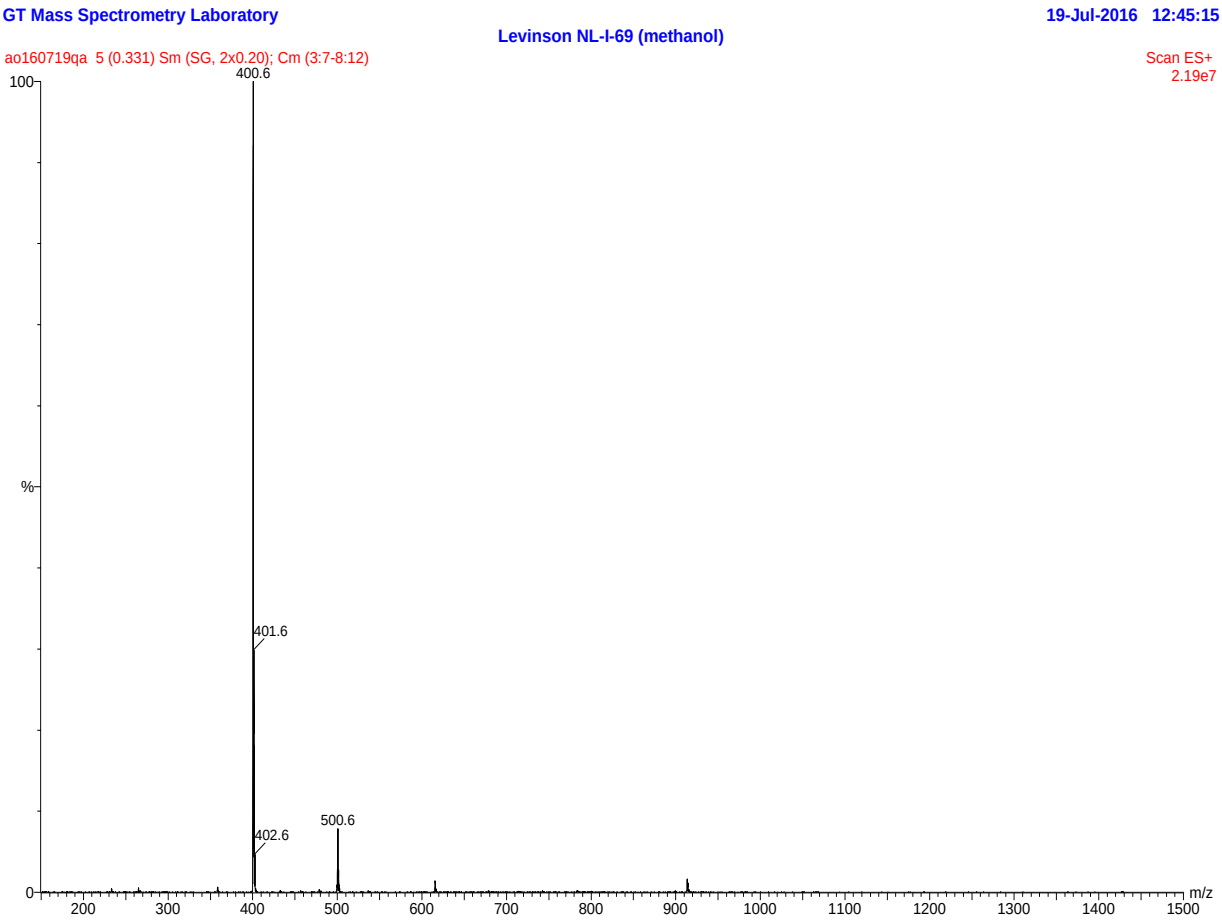


Figure B13 Mass spec of NL-I-69

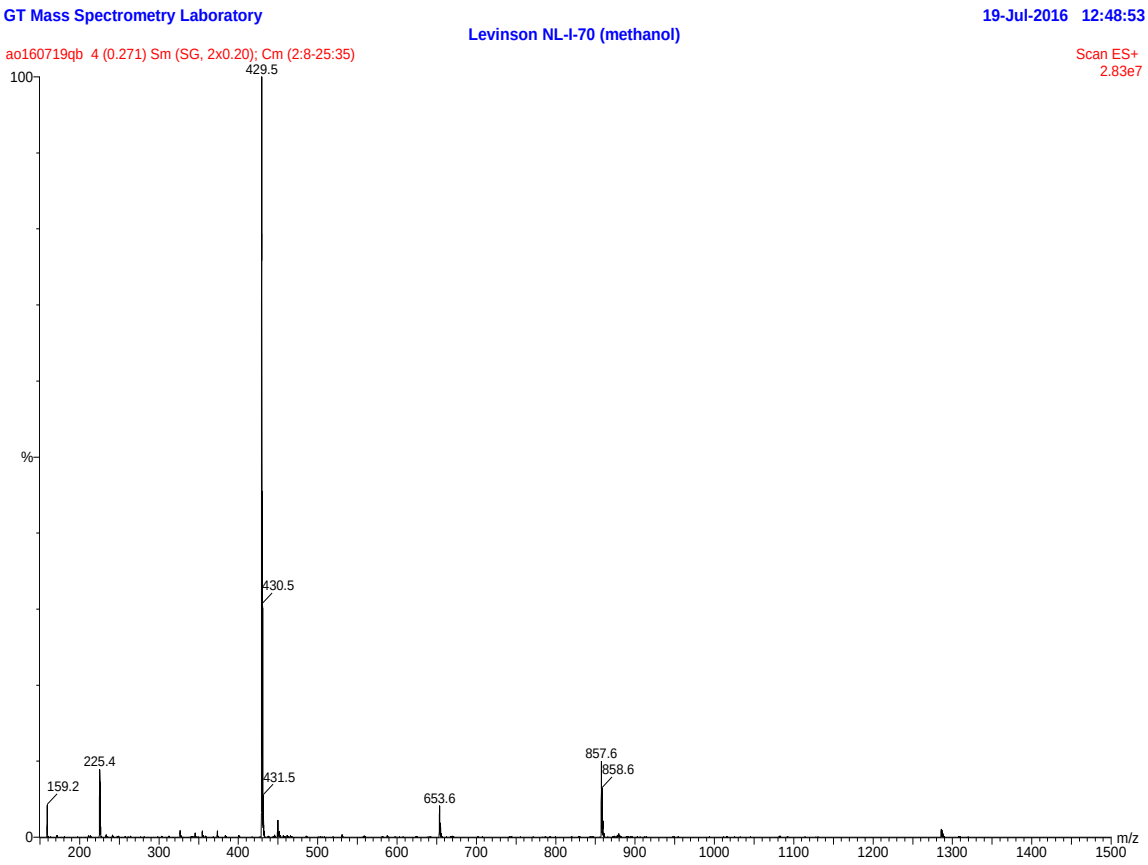


Figure B14 Mass spec of NL-I-70

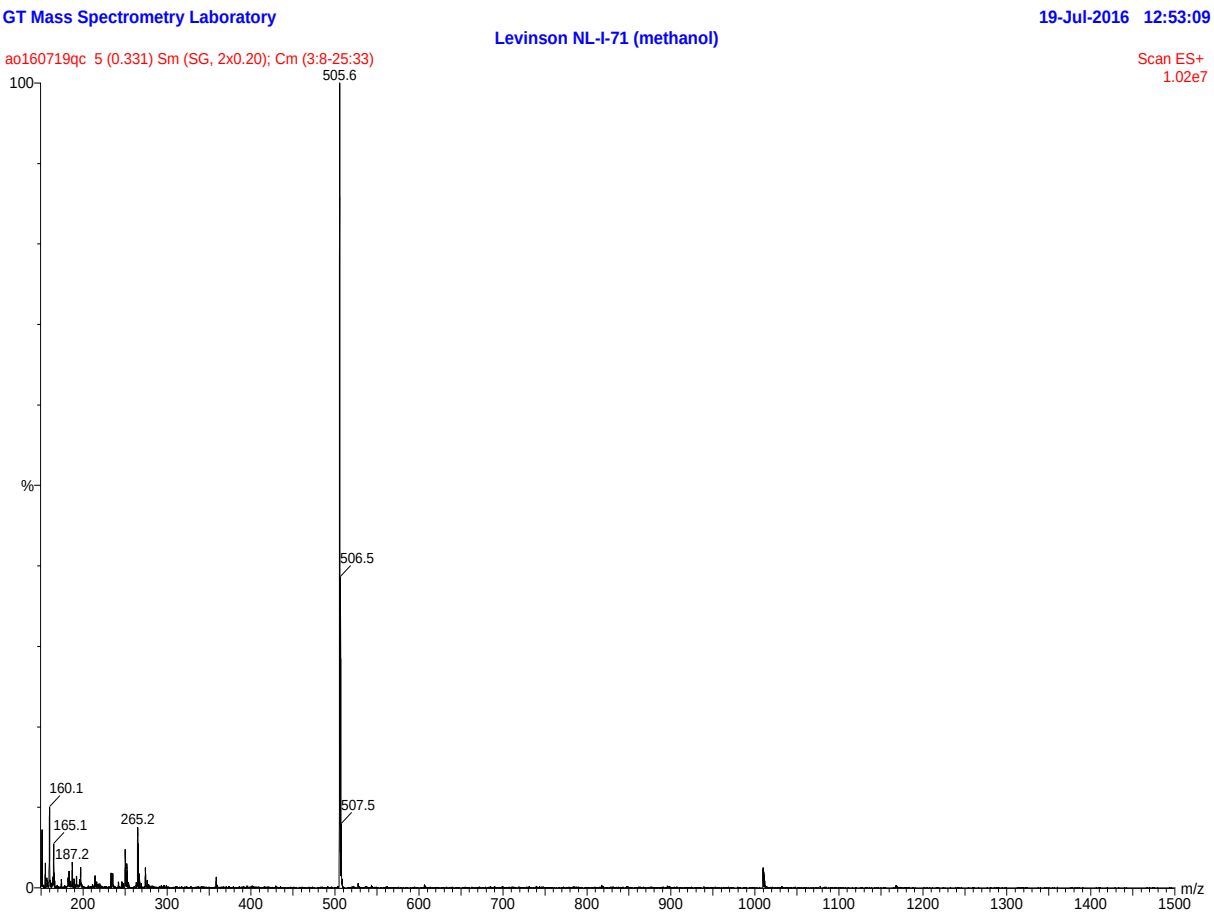


Figure B15 Mass spec of NL-I-71

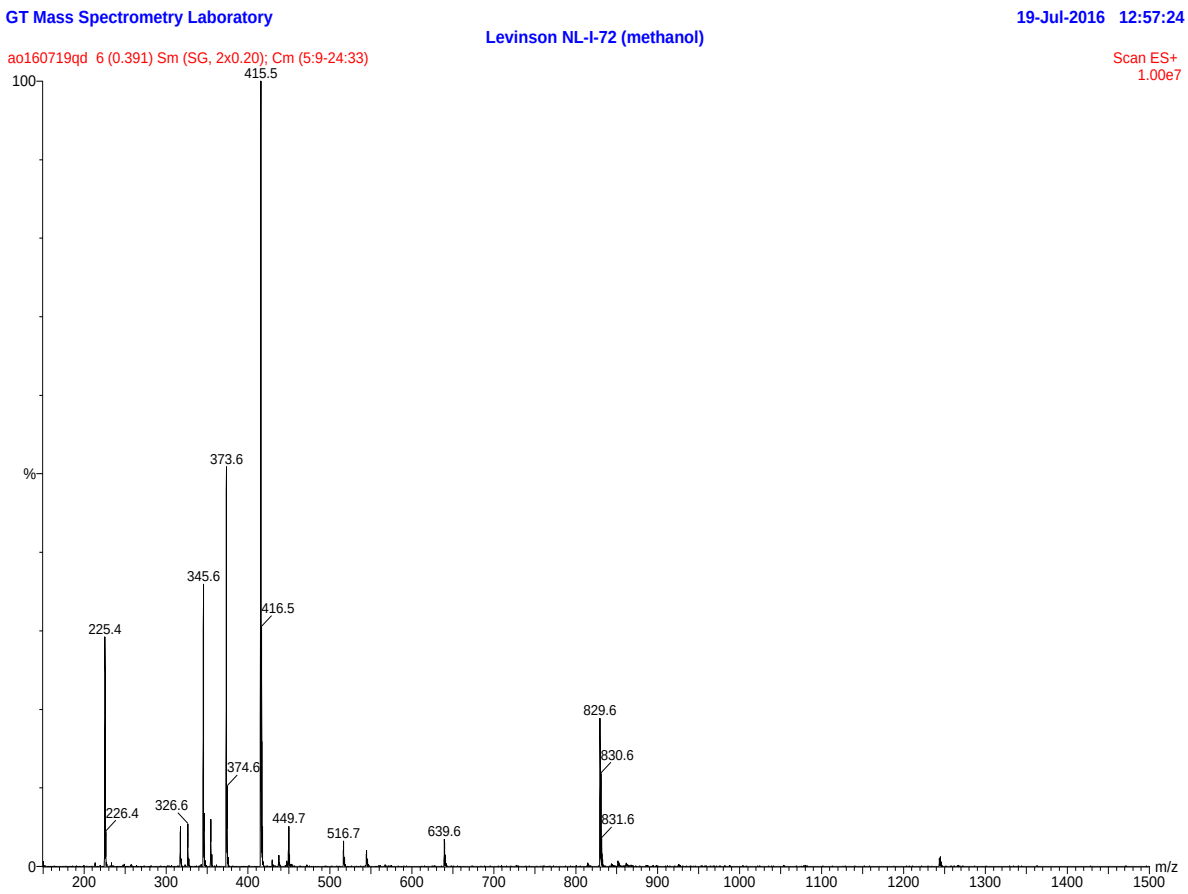


Figure B16 Mass spec of NL-I-72

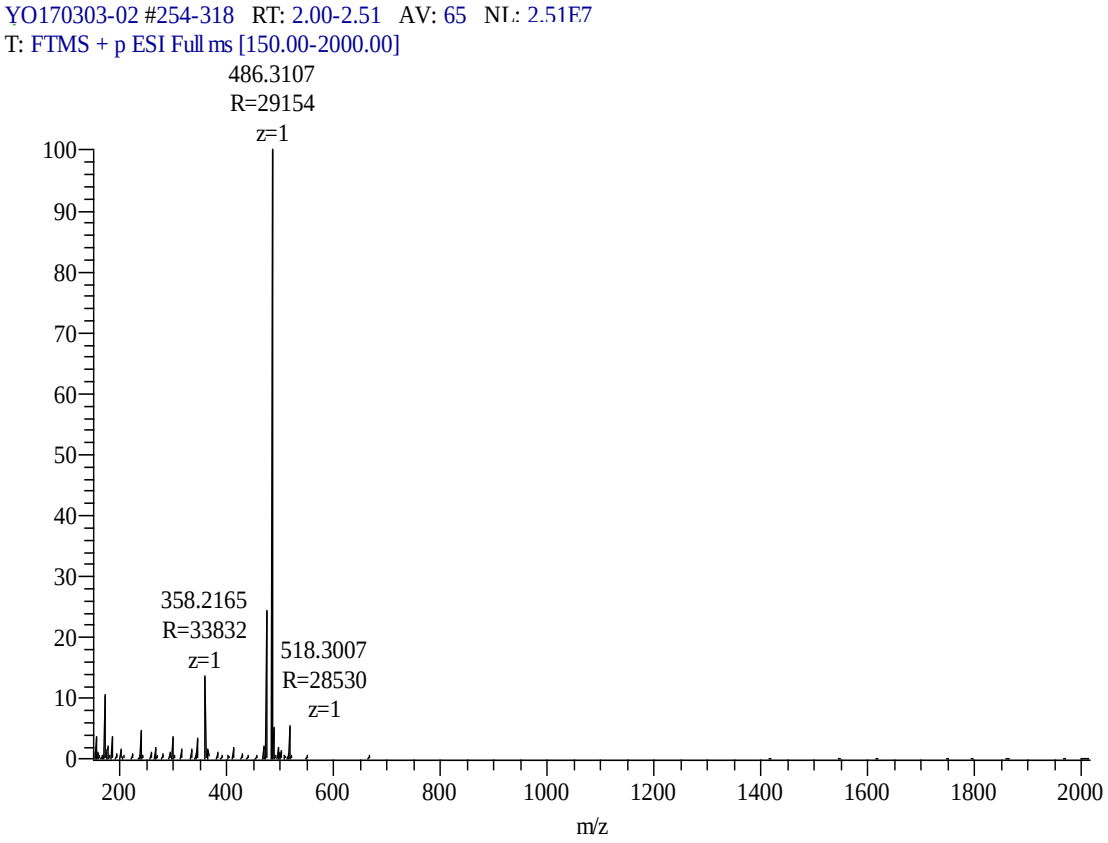


Figure B17 Mass spec of NL-I-97

APPENDIX C. MIC TRACES

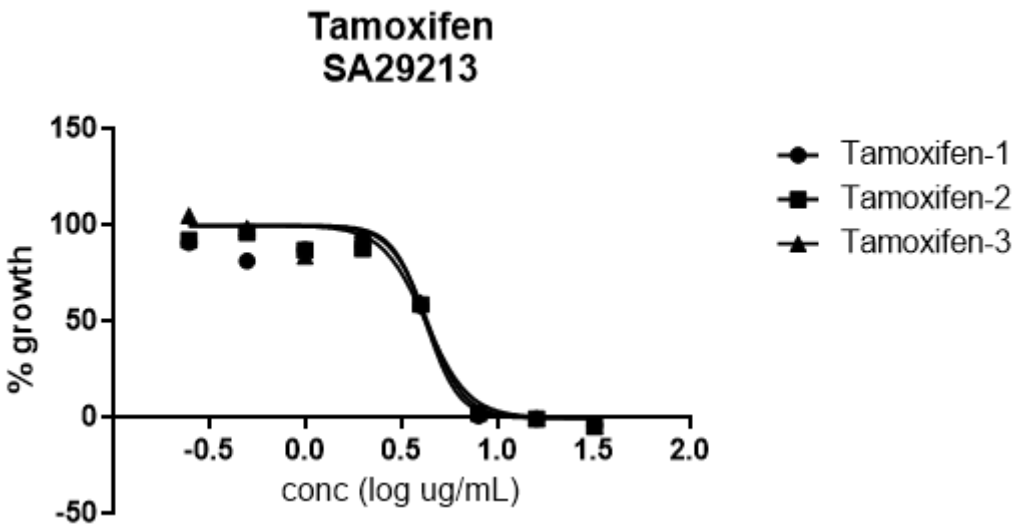


Figure C1 Tamoxifen effect on SA29213 growth

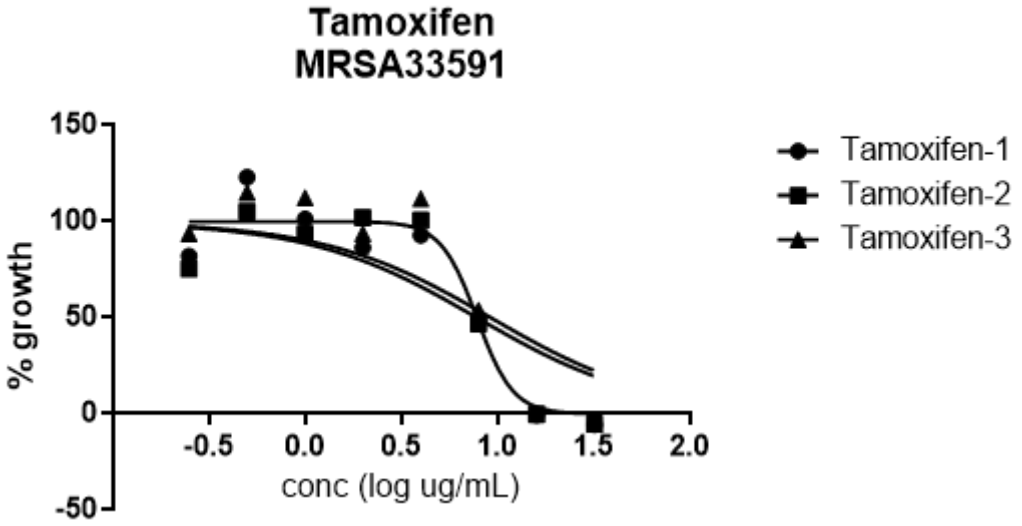


Figure C2 Tamoxifen effect on MRSA33591 growth

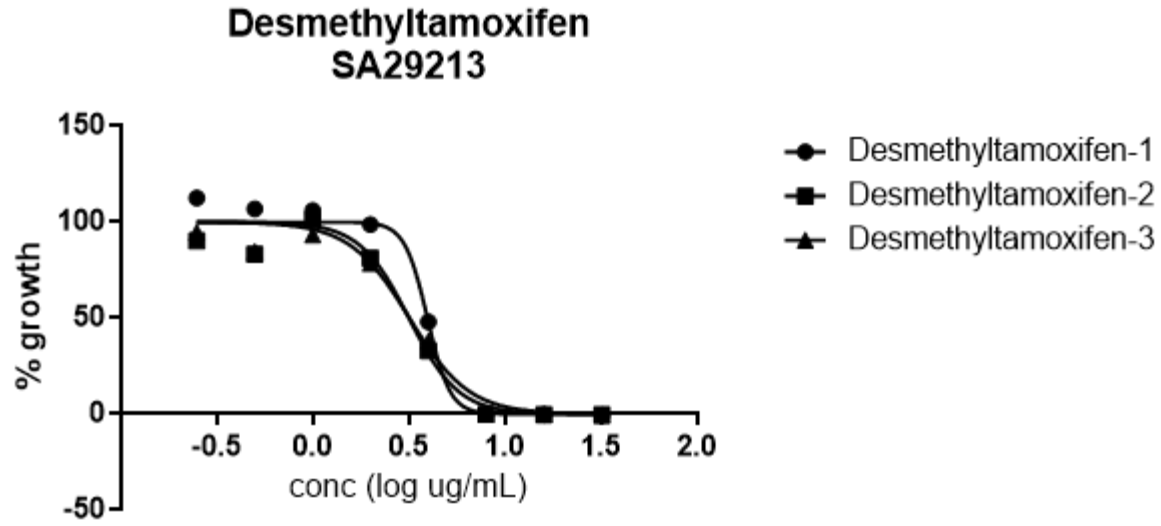


Figure C3 desmethyl tamoxifen effect on SA29213 growth

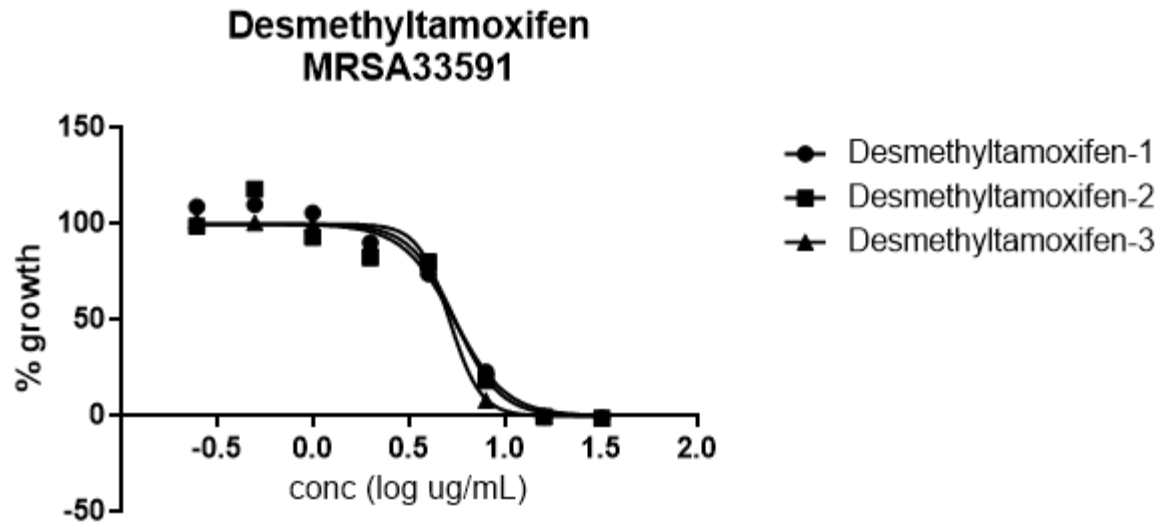


Figure C4 desmethyl tamoxifen effect on MRSA33591 growth

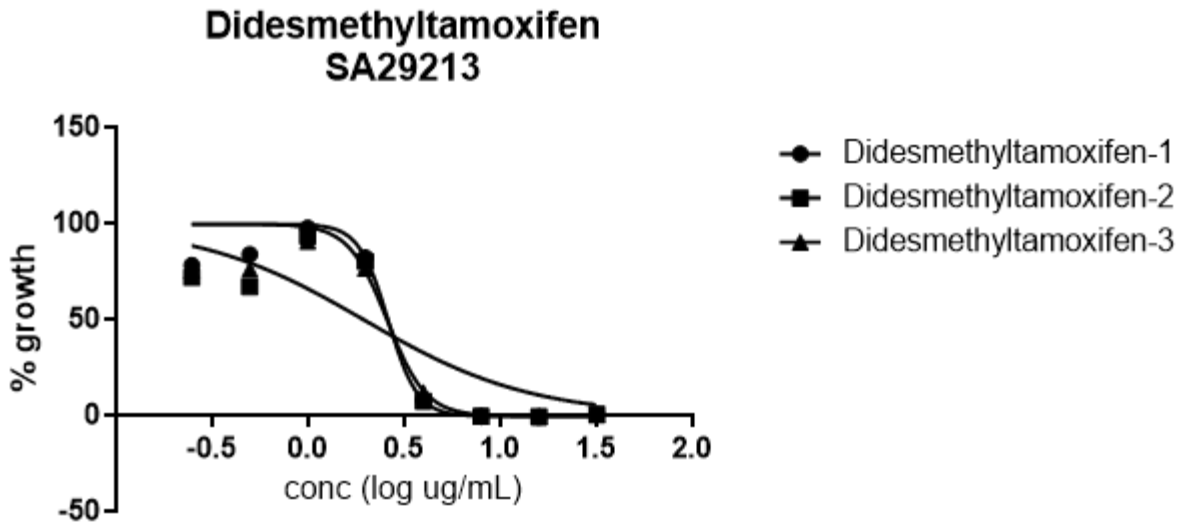


Figure C5 Didesmethyl tamoxifen effect on SA29213 growth

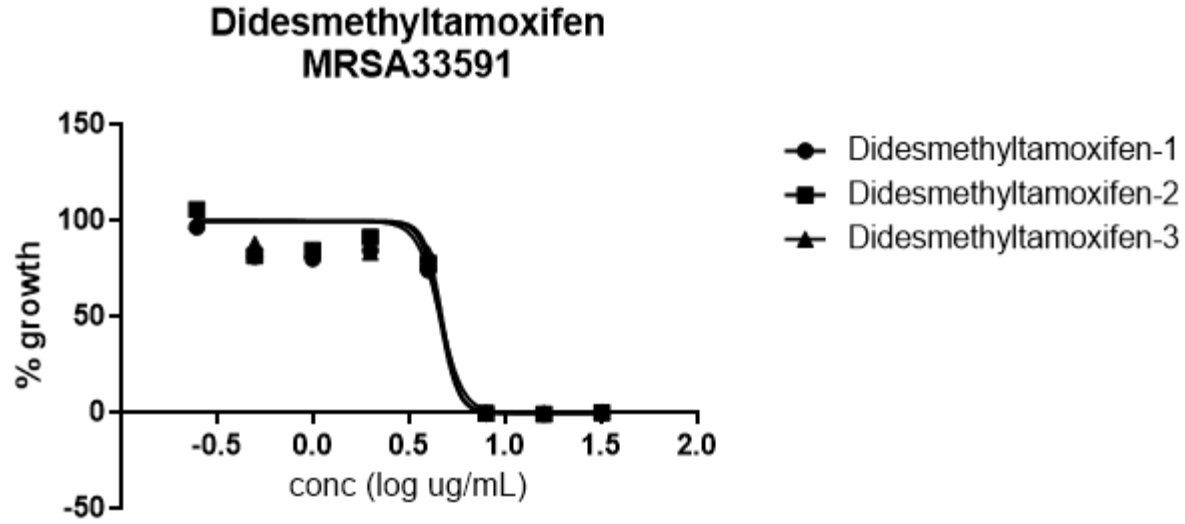


Figure C6 Didesmethyl tamoxifen effect on MRSA33591 growth

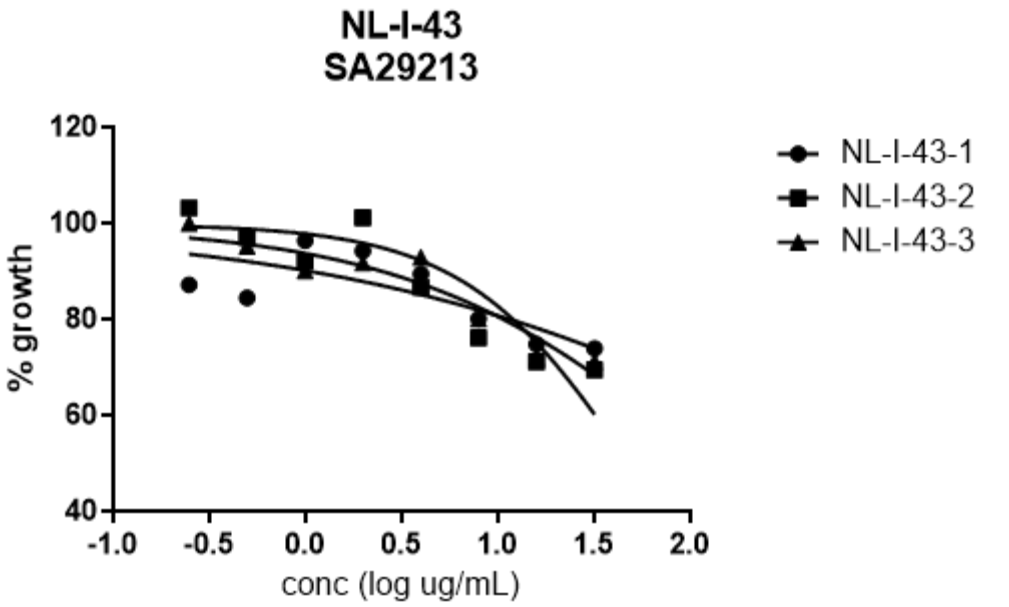


Figure C7 NL-I-43 effect on SA29213 growth

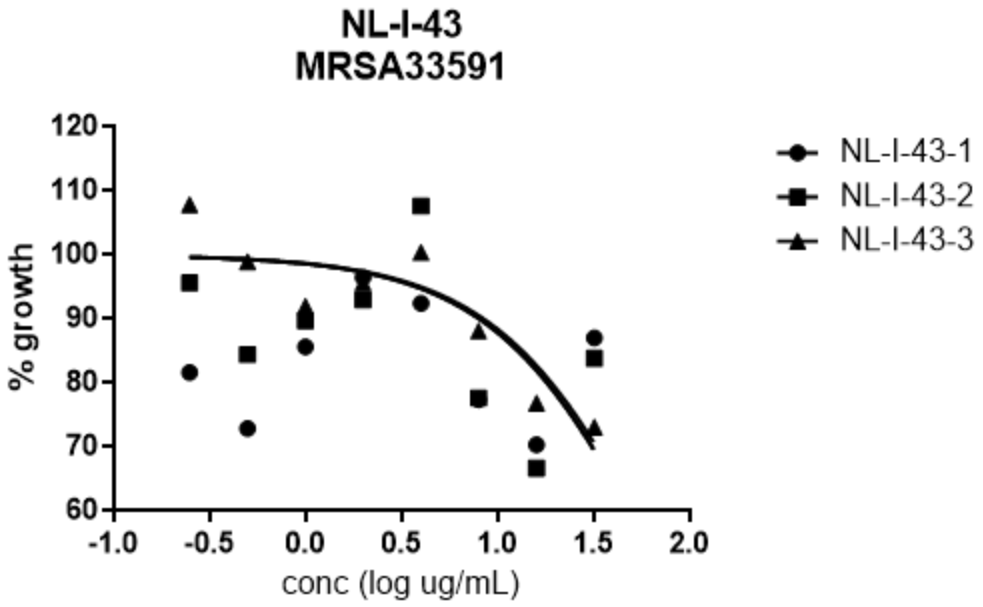


Figure C8 NL-I-43 effect on MRSA33591 growth

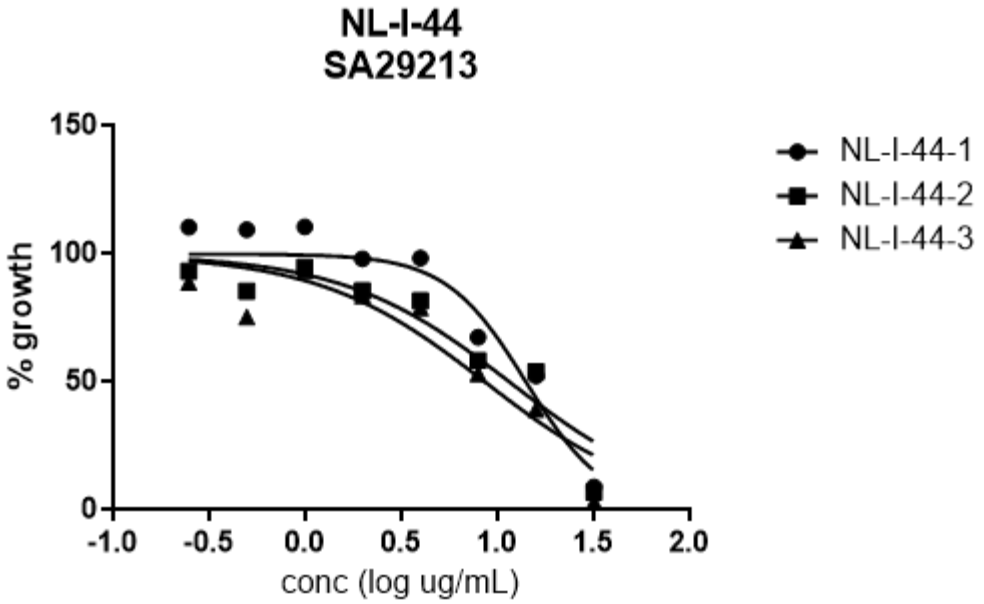


Figure C9 NL-I-44 effect on SA29213 growth

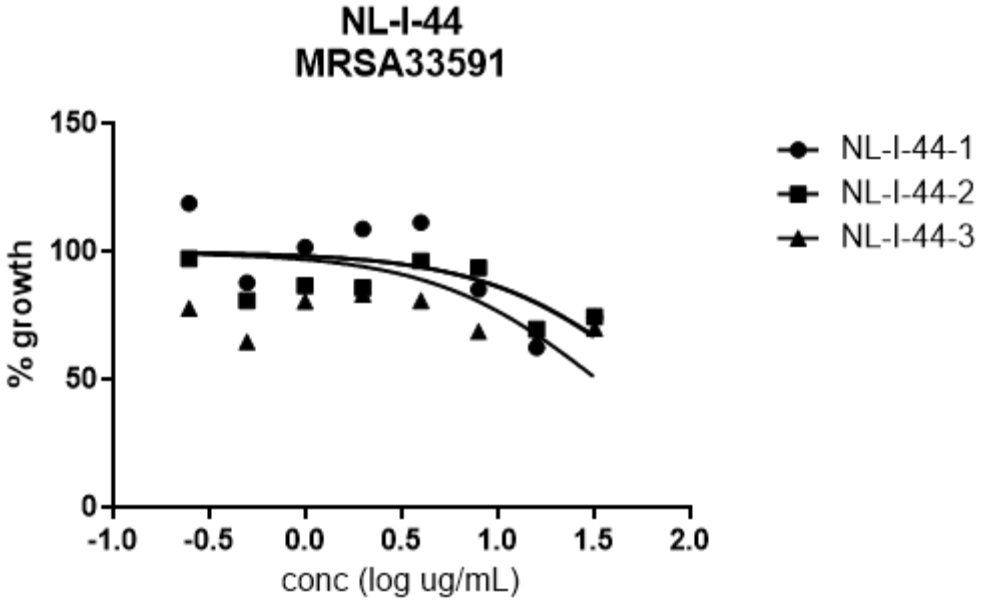


Figure C10 NL-I-44 effect on MRSA33591 growth

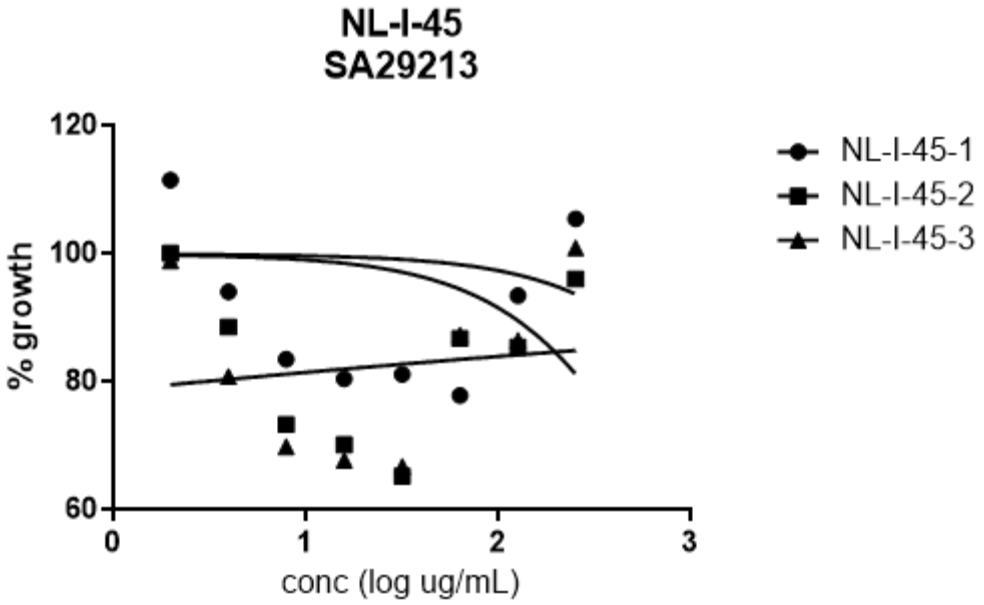


Figure C11 NL-I-45 effect on SA29213 growth

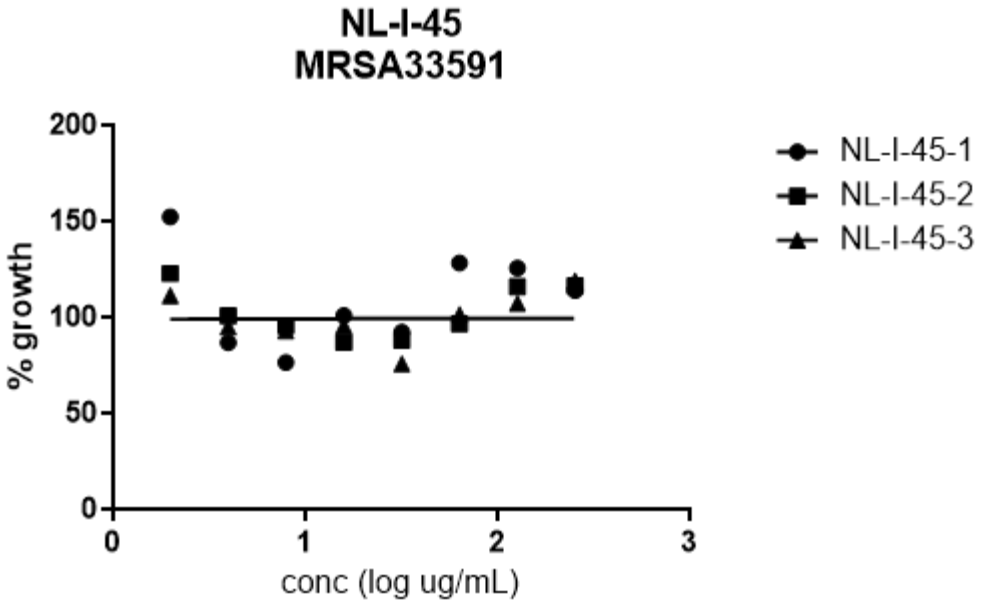


Figure C12 NL-I-45 effect on MRSA33591 growth

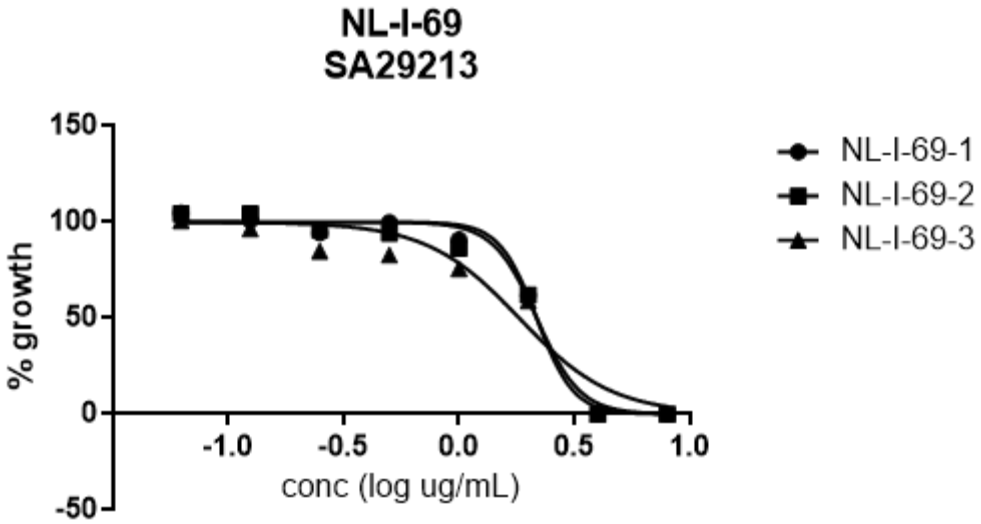


Figure C13 NL-I-69 effect on SA29213 growth

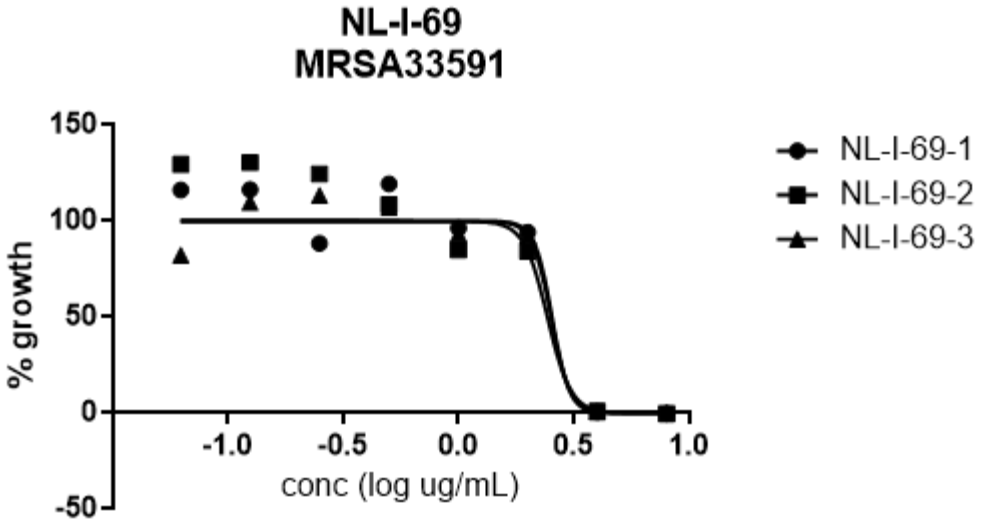


Figure C14 NL-I-69 effect on MRSA33591 growth

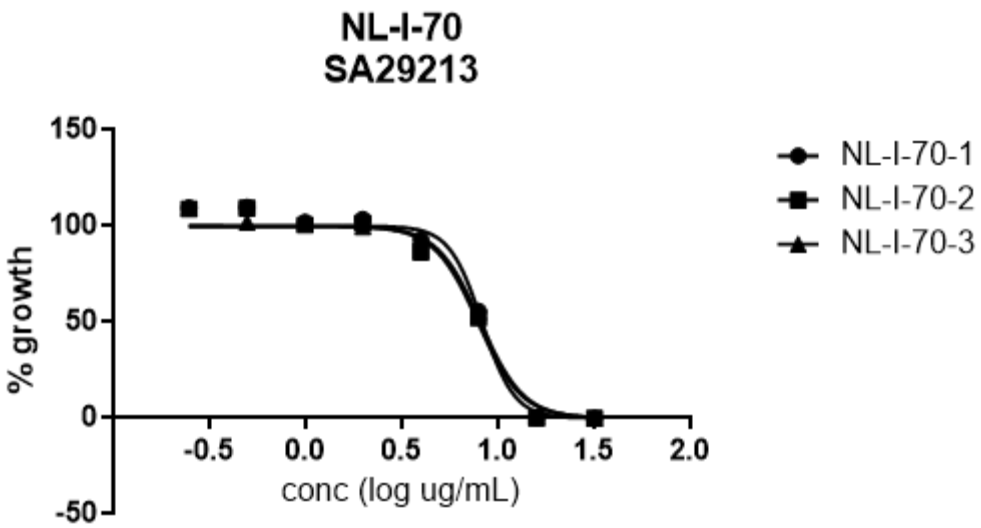


Figure C15 NL-I-70 effect on SA29213 growth

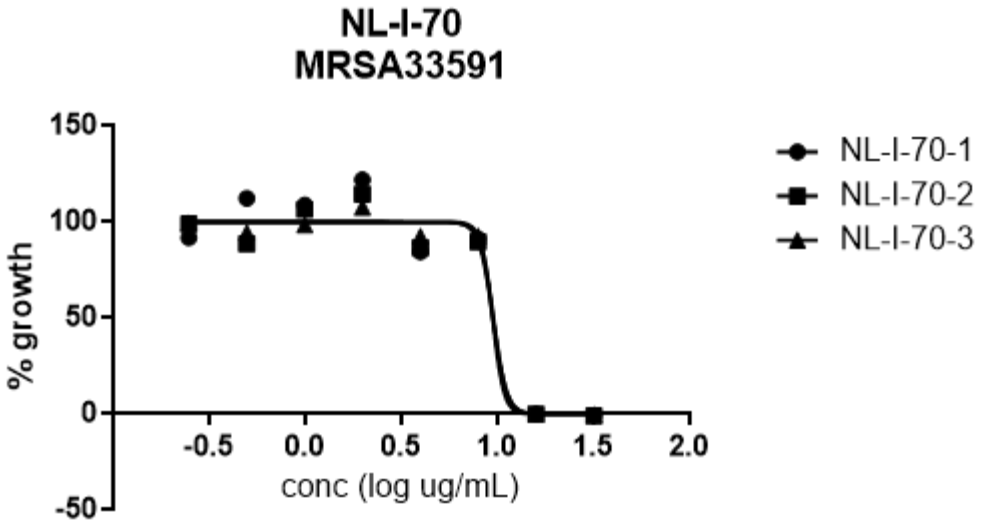


Figure 31NL-I-70 effect on MRSA33591 growth

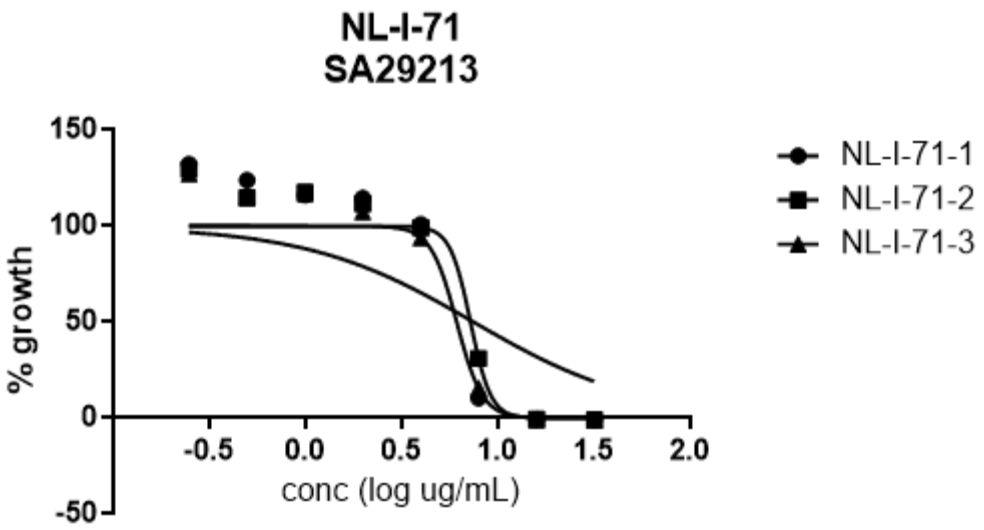


Figure C17 NL-I-71 effect on SA29213 growth

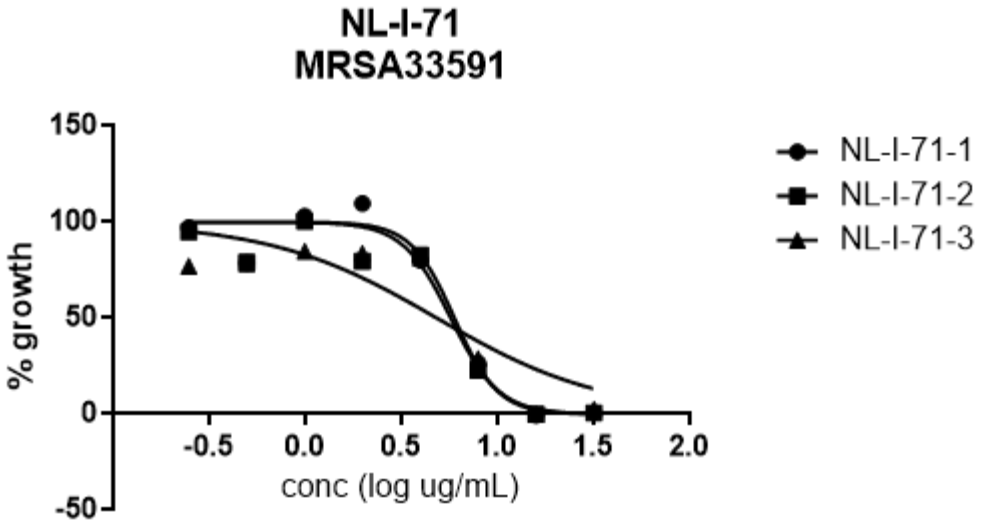


Figure C18 NL-I-71 effect on MRSA33591 growth

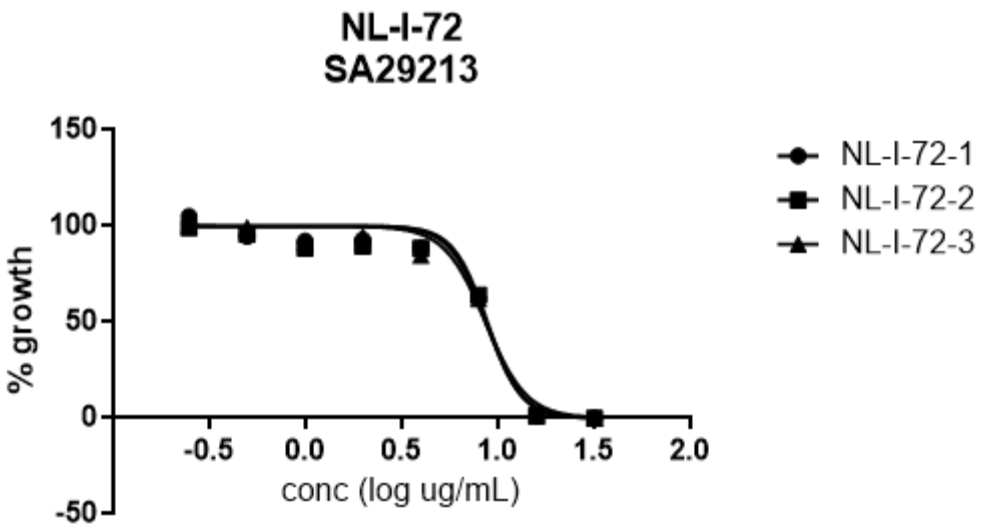


Figure C19 NL-I-72 effect on SA29213 growth

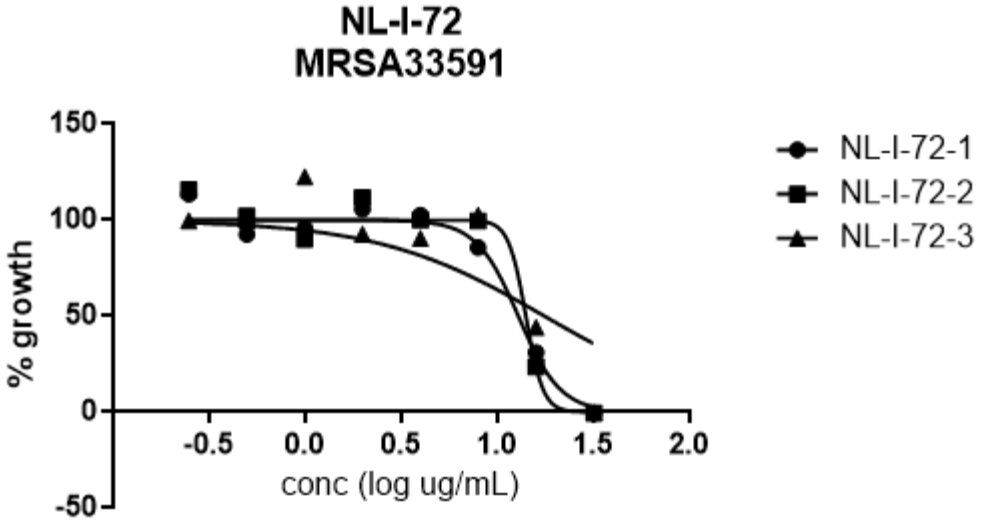


Figure C20 NL-I-72 effect on MRSA33591

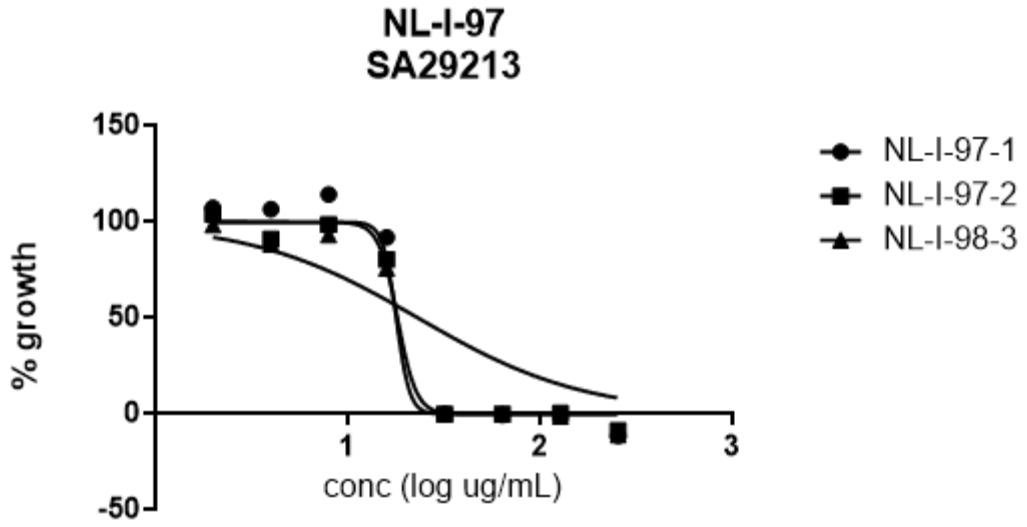


Figure C21 NL-I-97 effect on SA29213 growth

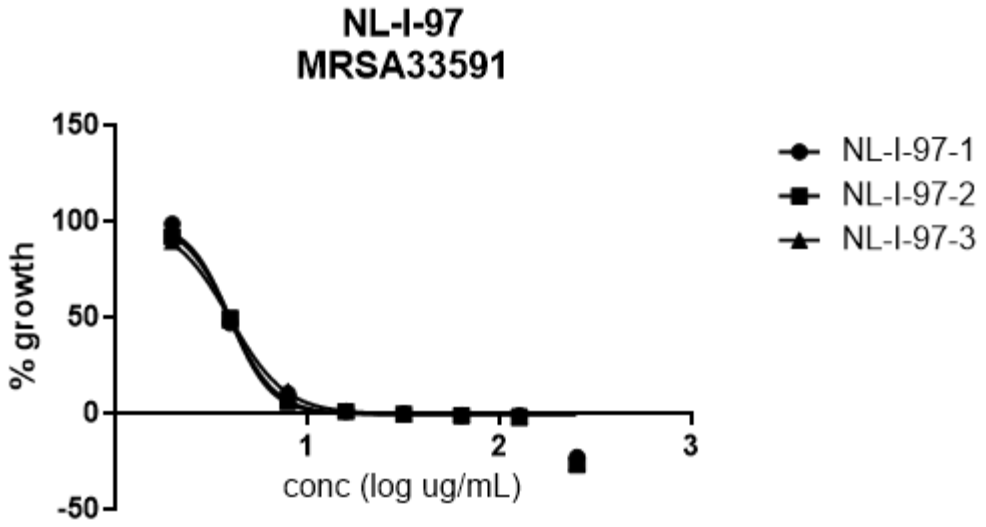


Figure C22 NL-I-97 effect on MRSA33591 growth

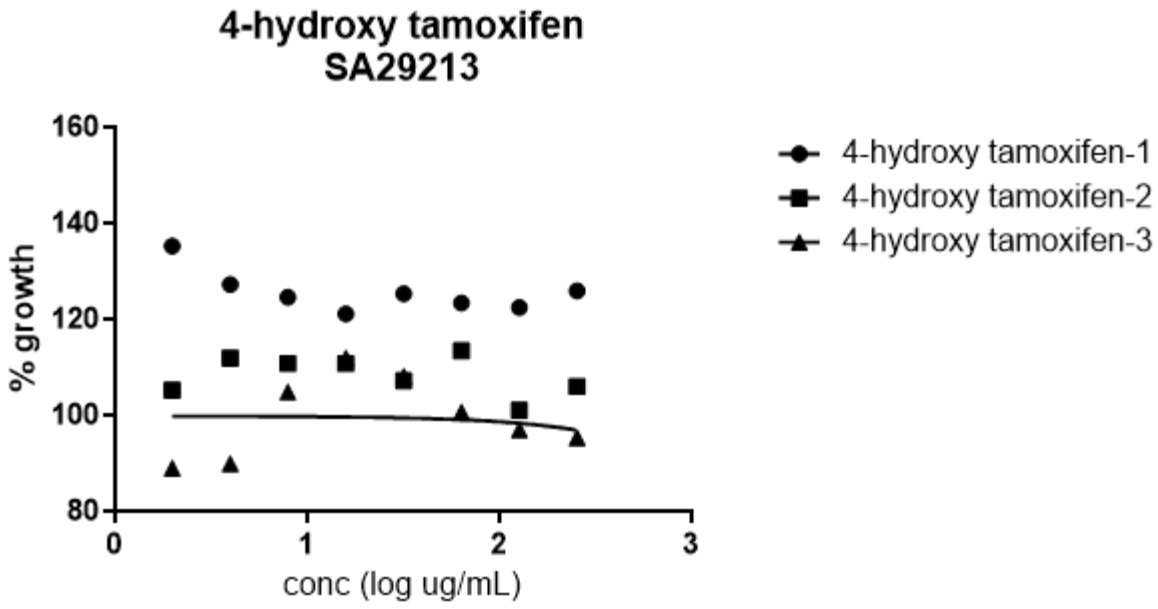


Figure C23 4-hydroxy tamoxifen effect on SA29213 growth

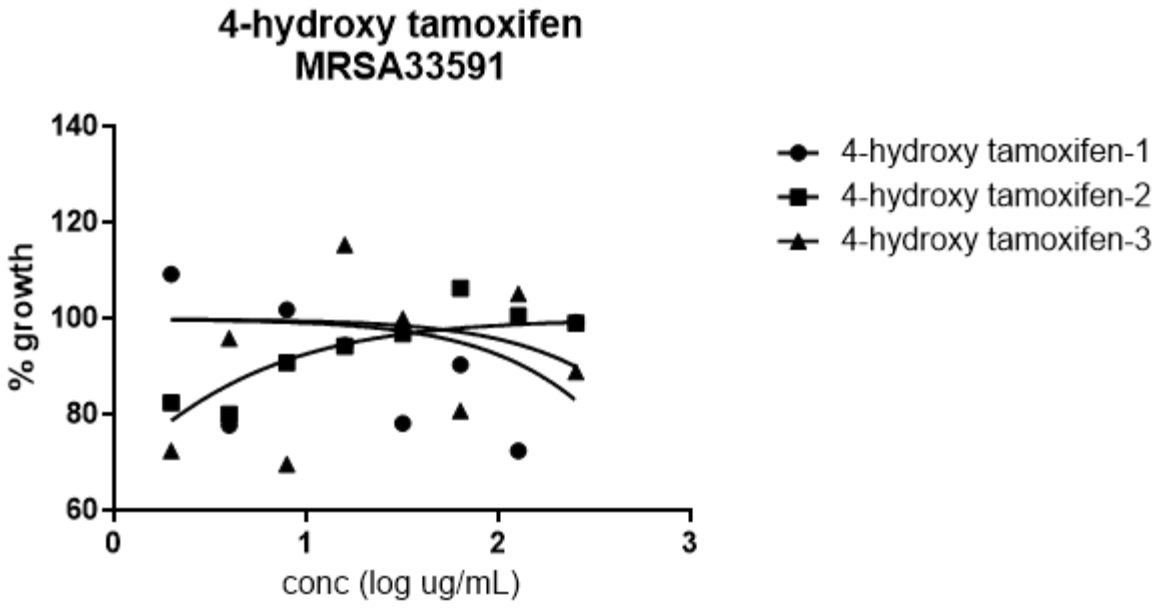


Figure C24 4-hydroxy tamoxifen effect on MRSA33591 growth

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